Gene silencing in plants: mechanisms & spread

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This thesis was submitted for the degree of Doctor of Philosophy of the Australian National University

Declaration

The research described in this thesis was conducted at CSIRO Plant Industry and the Australian National University between January 2001 and February 2004.

This thesis does not contain material which has been accepted for the award of any other degree or diploma. To the best of my knowledge, this thesis does not contain previously published material.

All work presented in this thesis is entirely my own except where clearly indicated in the text.

Jasmina Dedic-Hagan

October 2004

This thesis is dedicated to the memory of my grandmother

Sonja Kerouš

who gave me a wonderful childhood and laid a firm foundation

for my education

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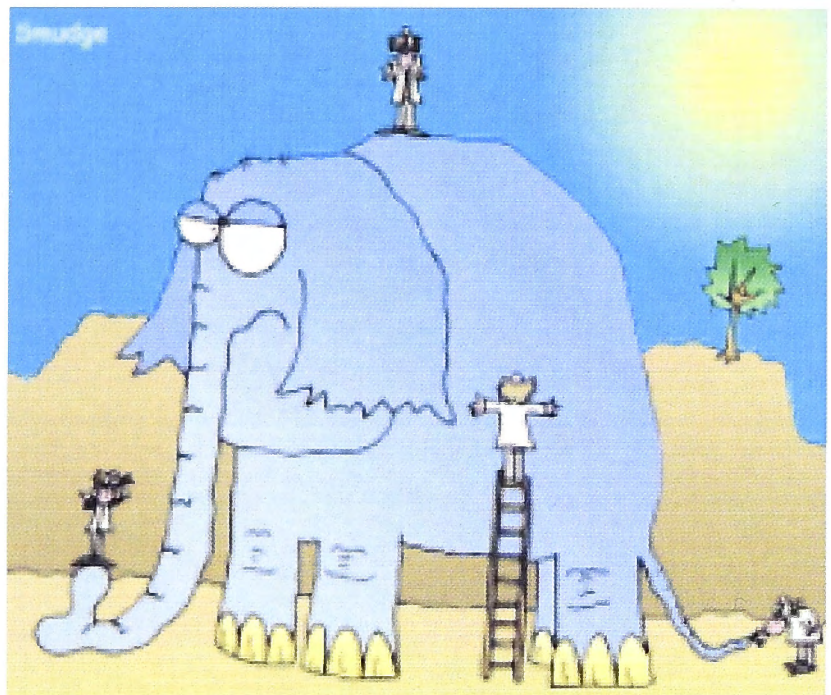
Abbreviations

5-Aza-C	5-azacytidine
ACE	ACTG-containing element
AGRF	Australian Genome Research Facility
AMV	alfalfa mosaic virus
AS	transgene with a sequence in anti-sense orientation
BAR	phosphinothricin acetyl transferase
bp	base pair
CC	companion cell
cdiGRP	cadmium induced glycine rich protein
CHS	chalcone synthase
CI	cylindrical inclusion protein
CMT	chromomethylase
CMV	cucumber mosaic virus
CP	coat protein
CymRSV	cymbidium ringspot virus
DDM1	decrease in DNA methylation 1
dFXR	*Drosophila* fragile X related protein
DNA	deoxyribonucleic acid
dNTP	deoxynucleoside triphosphate
DRM	domains re-arranged methyltransferase
dsRNA	double stranded RNA
EDTA	ethylenediamine tetra-acetic disodium salt
ELISA	enzyme linked immunosorbent assay
EMS	ethyl methanesulfonate
GFP	green fluorescent protein
GUS	β-glucuronidase
H3K9	histone 3 lysine 9
HC-Pro	helper component protease
HDAC	histone deacetylase
HMTase	histone methyltransferase
HP1	heterochromatin protein 1
HPLC	high pressure liquid chromatography

hpRNA	hairpin RNA
IPTG	isopropyl-β-D-thiogalactoside
kb	kilobase-pairs
kDa	kilodalton
KYP	kryptonite
LB	Leuria-Bertani medium
LHP1	like heterochromatin protein 1
MBD	methyl cytosine binding domain
Me	mouse ears mutation
MET1	methyltransferase 1
min	minute(s)
miRNA	micro RNA
MOM	morpheus molecule
MOPS	N-morpholino propane-sulfonic acid
MQ	mili-Q water
MRE	MYB recognition element
mRNA	messenger RNA
MS	Murashige-Skoog medium
NOS	nopaline synthase
NOSpro	nopaline synthase promoter
NPTII	neomycin phosphotransferase
nt	nucleotide
p30	30 kDa movement protein
PAL	phenylalanine lyase
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PDK	pyruvate orthophosphate dikinase
PDS	phytoene desaturase
PFD	pyrophosphate-dependant phosphofructokinase
PSTV	potato spindle tuber viroid
PTGS	post transcriptional gene silencing
PVX	potato virus X
PVY	potato virus Y
PVY-55N	potato virus Y - 55N strain

PVY-D	potato virus Y - strain D
PVY^N	necrotic potato virus Y strain
PVY^O	ordinary potato virus Y strain
RCNMV	red clover necrotic mosaic virus
RdDM	RNA directed DNA methylation
RdRP	RNA dependant RNA polymerase
RISC	RNA-induced silencing complex
RNA	ribonucleic acid
RNAi	RNA interference
RNase	ribonuclease
rpm	revolution per minute
RT	room temperature
RT-PCR	reverse transcriptase polymerase chain reaction
Rubisco	ribulose-1,5-bisphosphate carboxylase/oxygenase
S	transgene with a sequence in sense orientation
s	second(s)
S+AS	sense and antisense transgenes
SAM	s-adenosylmethionine
SDS	sodium dodecyl sulphate
SE	sieve element
SEL	size exclusion limit
siRNA	small interfering RNA
ssRNA	single stranded RNA
TBE	Tris-borate-EDTA
TE	Tris-EDTA
TEV	tobacco etch virus
TGB	triple gene block
TGMV	tomato golden mosaic virus
TGS	transcriptional gene silencing
TLC	thin layer chromatography
TMV	tobacco mosaic virus
TRV	tobacco rattle virus
TSWV	tomato spotted wilt virus
tt	transparent testa mutation

U	unit
UV	ultraviolet
VIG	vasa intronic gene protein
VIGS	virus induced gene silencing
X-gal	5-bromo-4-chloro-3-indoyl-β-D-galactopyranoside
X-gluc	5-bromo-4-chloro-3-indoyl-β-D-glucuronic acid



"Imagine three researchers locked in a dark room with an elephant. One grasping the trunk, decides it is a fire hose. The second, holding a leg, is sure it is a tree. The third, feeling the contour of an ear imagines it is an uncooked pie crust. Then someone flicks the light switch, and all is revealed.

That, more or less, has been the story of one of the hottest new areas in biology – gene silencing"

News feature
Nature, Vol 404, April 2000

Abstract

Abstract

Plants have intrinsic and sequence-specific RNA degradation and DNA methylation mechanisms that are triggered and directed by double-stranded (ds) RNA. These mechanisms can be triggered by transgenes encoding dsRNA or self-complementary single stranded "hairpin" (hp) RNA to give specific gene silencing or virus resistance.

Tobacco plants transformed with transgenes encoding either dsRNA or hpRNA that were derived from the genome of potato virus Y (PVY) were analysed for their resistance against a suite of PVY strains. Both types of transgene conferred robust protection against a range of related virus strains. The minimum length of sequence identity required for effective transgene-mediated virus resistance was 20 nucleotides. The resistance was more rapid in plants containing the hpRNA transgenes than in plants expressing transgenic dsRNA.

Cross-protection is a well-known phenomenon whereby the infection of a plant by a mild virus strain protects it from subsequent infection by a severe, but related, strain. This study demonstrated that cross protection and transgene-mediated protection provide resistance to a similar range of viral strains, suggesting that both strategies rely on the same basic gene silencing mechanism. However, an *in planta* expressed suppressor of gene silencing, HC-Pro, was able to inhibit transgene mediated protection, but not cross-protection. This raises the possibility that cross-protection and transgene-mediated protection operate through different branches of the silencing pathway.

Although dsRNA and hpRNA transgenes showed similar responses to viral challenge, only the hpRNA was able to generate a signal that was able to establish systemic silencing. The spread of silencing was unidirectional, traveling from the rootstock to the scion. The spread of silencing was only effective when the scion was derived from very young plants and when there was 100% identity between the inducing hpRNA and its distal target.

The spread of silencing through a plant suggests that amplification of the silencing signal occurs, probably both at the site of generation and at the site of action. To test this hypothesis, sections of the endogenous chalcone synthase gene were targeted for silencing by hpRNA transgenes and examined in detail. DNA methylation, a molecular hallmark of gene silencing, was found at high levels at the chalcone synthase gene in silenced plants. High levels of DNA methylation were restricted to within 100 nucleotides of the targeted sections of the chalcone synthase gene, suggesting that primed amplification did not occur. A co-operative DNA/histone modification model is proposed that could account for these findings.

Chapter 1

Introduction

1.1 General introduction

The central dogma of molecular biology is that DNA controls metabolism by directing cells to make specific proteins via RNA templates. In this model, RNA is envisaged as a simple mobile link between the information-containing DNA and a function-performing protein. However, the discovery of catalytic RNAs (Cech 1986, Forster & Symons 1987, Hampel & Tritz 1989) and the identification of small RNAs as mediators of many essential cellular procecsses (Waterhouse *et al.* 2001, Wassenegger 2002, Stokes 2003, Stevenson & Jarvis 2003, Carrington & Ambros 2003), challenged this view. It is now apparent that RNAs perform a diverse array of essential functions within a cell. They serve not only as information carrying molecules but also as structural, catalytic and regulatory molecules.

One kind of RNA species called micro RNAs (miRNAs) have been shown to regulate developmental processes by controlling translation. Small double stranded RNAs have been implicated in a wide range of processes including transgene silencing (Mette *et al.* 2000), plant defence against viruses (Waterhouse *et al.* 2001) and genome maintenance (Dernburg & Karpen 2002, Volpe *et al.* 2003). It has become clear that RNAs can perform these functions not only in cells that produce them but also in other distal cells (Fagard & Vaucheret 2000, Kim *et al.* 2001).

Recently, new insights have been gained into the formation and regulatory mechanism of double stranded RNAs. Double stranded RNA-based silencing technology is now routinely utilised by researchers to regulate gene expression in a wide range of organisms and has potential applications in basic science, agriculture and medicine.

1.2 Discovery of gene silencing

Over the past three decades, genetic engineering technology has been successfully used to introduce desired traits into a wide variety of organisms. This process involves the introduction of additional copies of an endogenous gene, or a gene originating from a different species, into the genome of the target organism. However, a proportion of transgenic progeny do not display the desired trait. Moreover, in some instances, endogenous genes with homology to the transgene become silenced. In genetic engineering experiments, organisms that displayed these phenomena were often discarded and the underlying reasons for the gene silencing were never investigated. However, a series of accidental discoveries indicated that gene silencing results from the action of an ancient RNA-mediated mechanism that has evolved to control the level of specific endogenous mRNAs and foreign, invading RNAs.

One of the first studies of plant gene silencing resulted from an attempt to enhance flower pigmentation by the introduction of a chalcone synthase (*CHS*) transgene in petunia plants. The *CHS* gene encodes an enzyme involved in the anthocyanin pigment pathway and its over-expression in transgenic petunia was expected to result in elevated levels of anthocyanins and a dark purple flower colour. However, a proportion of the transgenic plants produced totally white or patchy-white flowers (Napoli *et al.* 1990, van der Krol *et al.* 1990), indicating that anthocyanin levels had actually been reduced in these plants. One possible explanation for this result was that both the endogenous *CHS* gene and the transgene had been silenced.

Similarly puzzling observations were made by researchers attempting to engineer virus resistance in transgenic plants by expressing genes for viral coat proteins (Lindbo *et*

al. 1993). In this case, virus resistance was often achieved without any detectable transgenic protein (Smith *et al.* 1994, Guo & Garcia 1997). Subsequent research demonstrated that virus resistant plants, as well as the *CHS*-silenced plants, exhibited high levels of transgene transcription followed by the rapid degradation of transgene RNA (Napoli *et al.* 1990, van der Krol *et al.* 1990, Lindbo *et al.* 1993, Smith *et al.* 1994). Moreover, any homologous RNA, be it endogenous or viral, was also targeted for degradation (Napoli *et al.* 1990, Mueller *et al.* 1995, Goodwin *et al.* 1996). This phenomenon was named post-transcriptional gene silencing (PTGS).

Researchers working with *Drosophila melanogaster* and *Neurospora crassa* also reported a negative correlation between transgene copy number and gene expression (Romano & Macino 1992, Cogoni *et al.* 1994, Pal-Bhadra *et al.* 1999). This gene silencing phenomenon applied to both the transgenes themselves and to highly homologous endogenous genes and operated at the post-transcriptional level. When the transgene copy number was reduced, gene expression was re-established (Cogoni *et al.* 1994). However, the molecular mechanism responsible for the silencing of transgenes and the co-suppression of the related genes was unknown at this stage.

The phenomenon called RNA interference (RNAi) was first described by Fire *et al.* (1998). This group was attempting to interfere with the expression of endogenous genes in *Caenorhabditis elegans* by injecting antisense RNA complementary to the target mRNA. This approach resulted in a degree of gene silencing but was no more effective than the control molecule, a translatable sense RNA. The authors hypothesised that aberrant double-stranded molecules present at low levels in their RNA preparations were responsible for the observed silencing. To investigate this

further, worms were injected with highly purified sense or antisense or annealed sense + antisense preparations. The authors found that sense or antisense preparations alone caused minimal interference and only when injected at high concentrations. In contrast, even very small amounts, a few molecules per cell, of the double stranded preparation were able to induce potent and specific silencing. Moreover, gene silencing could be induced by incubating the worms in dsRNA preparations, feeding them bacteria expressing dsRNA, or by introducing dsRNA-producing gene constructs into the worms themselves (Timmons & Fire 1998, Tabara *et al.* 1998, Tavernarakis *et al.* 2000).

Waterhouse *et al.* (1998) were first to propose that dsRNA is able to induce gene silencing in plants. The authors generated *Nicotiana tabaccum* plant lines expressing an untranslatable NIa protease gene from potato virus Y (PVY) in sense (S) and antisense (AS) orientations. Neither of these transgenes, on their own, was able to induce silencing of PVY genes. As such, plants expressing either S or AS transgene were susceptible to PVY. However, plants expressing both S and AS transgenes, termed S+AS plants, were found to be immune to PVY. The authors proposed that the S and AS transcripts anneal to form dsRNA which then mediates post transcriptional silencing of homologous sequences (Waterhouse *et al.* 1998).

Plant researchers observed that gene silencing could also occur when, in the same plant, one type of promoter is used to drive the expression of multiple independent transgenes encoding different genes. In this case however, silencing was due to the loss of transcription and was therefore termed transcriptional gene silencing (TGS) (Vaucheret 1993, Park *et al.* 1996). Detailed analysis of these silenced plants revealed

that the transgenes were often present in multiple copies at a single locus. In addition, the transgene promoters were found to be heavily methylated. When additional promoter sequences were introduced into the silenced plants by classical breeding or super-transformation, the new promoter sequences quickly became silenced and heavily methylated (Vaucheret 1993, Matzke *et al.* 1994, Mette *et al.* 1999). Subsequent studies showed that in many cases TGS, like PTGS, was mediated by dsRNA. PTGS was mediated dsRNA-guided cleavage of the homologous transcript, while TGS was initiated by the dsRNA-guided methylation of homologous promoter sequences (Waterhouse *et al.* 1998, Smith *et al.* 2000, Chuang & Meyerowitz 2000, Mette *et al.* 2000).

1.3 The role of gene silencing

In plants, post-transcriptional gene silencing is likely to have evolved as a protective mechanism against viral infections. Rapid and readily observable gene silencing in plants follows infection by a number of virus genera including nepoviruses, caulimoviruses and tobraviruses (Covey *et al.* 1997, Ratcliff *et al.* 1997, Ratcliff *et al.* 1999). In cauliflower mosaic virus-infected *Brassica oleracea gongylodes*, the virus initially infects the plant and spreads systemically. However, approximately three weeks post infection, the emerging leaves are symptom-free and exhibit homology-dependent resistance to secondary infection by the same virus. Covey *et al.* (1997) demonstrated that the non-symptomatic leaves supported viral replication but rapidly degraded any viral RNA.

Experiments using recombinant viruses and transgenic plants have provided important insights into the relationship between viral infection and post-transcriptional silencing.

Recombinant viruses carrying sequences of plant genes or transgenes have been shown to induce silencing of homologous plant genes and transgenes (Al-Kaff *et al.* 1998, Ratcliff *et al.* 1999). For example, tobacco mosaic virus (TMV) carrying a segment of the plant phytoene desaturase (*PDS*) gene, induced silencing of *PDS* in infected plants. These plants accumulated high levels of phytoene and displayed a bleached phenotype, typical of PDS dysfunction (Kumagi *et al.* 1995). In addition, transgenic plants with pre-established PTGS showed resistance to recombinant viruses with sequence homology to the silenced gene. For example, recombinant potato virus X (PVX), encoding a segment of the *GUS* gene, was rapidly degraded in plants with post-transcriptionally silenced *GUS* transgene (English *et al.* 1996). The discovery of a viral suppressors of PTGS provided further evidence that RNA mediated gene silencing plays a role in a plant's defence against viruses (Marathe *et al.* 2000a). These suppressors have been found in numerous viruses (Voinnet *et al.* 1999), suggesting that they have evolved to combat PTGS-based plant defences.

PTGS also appears to function as a defence system against transposons, mobile genetic elements that randomly insert themselves in the genome. Some transposons are self-excising and jump from one genomic location to another. However, the majority of transposons use replicative transposition, which involves the synthesis of additional transposon copies (Campbell & Reece 2002). Such transposons replicate via an RNA intermediate, which encodes enzymes that mediate the transposition process (Fig. 1.1).

Replicative transposition often results in the accumulation of several thousand transposon copies within a genome. Such transposon activation can have a range of effects including alterations in endogenous gene expression, gene deletion or insertion

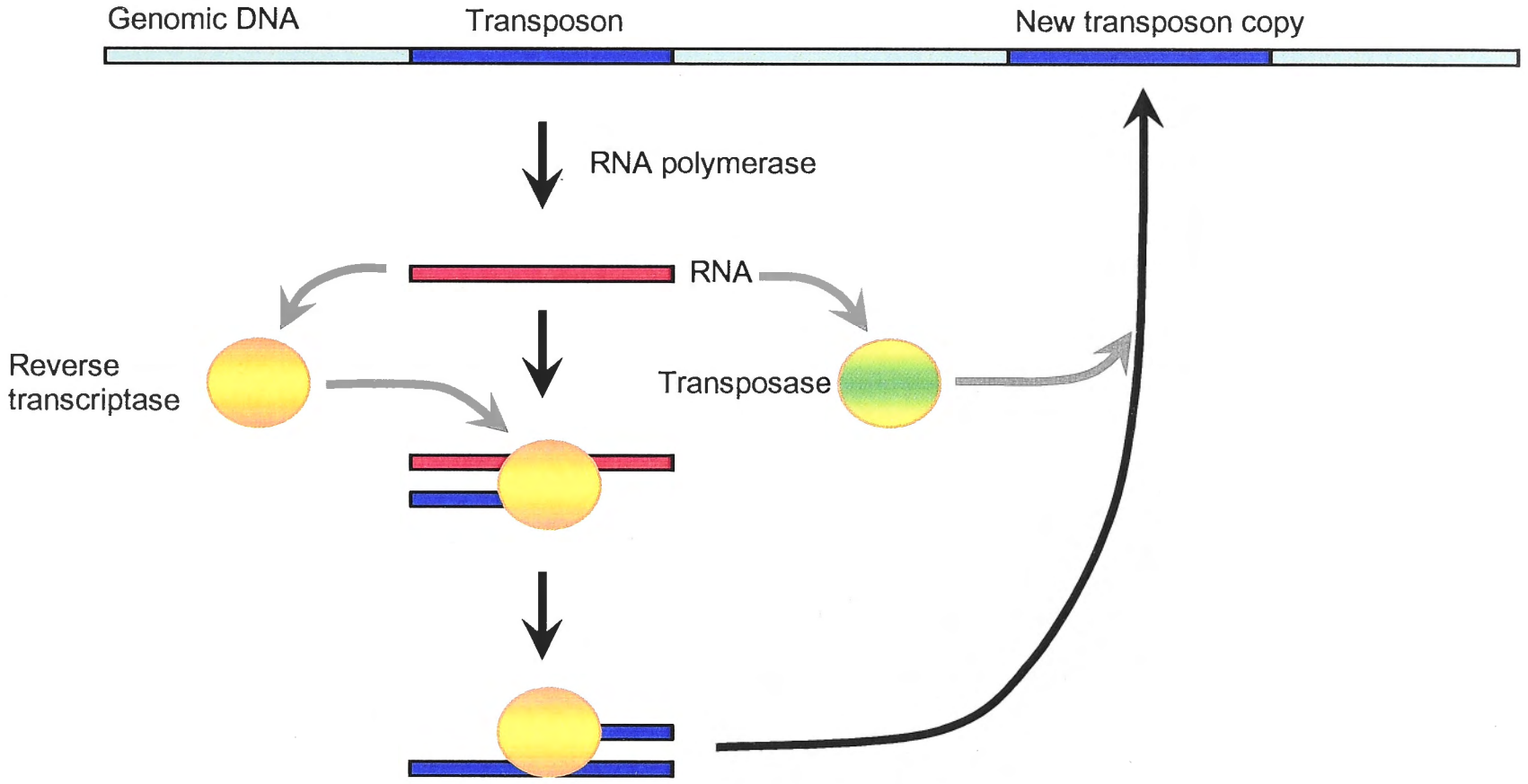


Figure 1.1: Transposon replication and insertion. (adapted from Campbell and Reece 2002)
Retrotransposon DNA is transcribed by the host RNA polymerase. Translation of the retrotransposon RNA yields reverse transcriptase and transposase enzymes. Reverse transcriptase catalyses synthesis of a DNA strand from the retrotransposon RNA template while transposase catalyses the transposition to a new site in the genome.

and chromosome rearrangements. As these changes are usually deleterious, the inactivation of transposons can be crucial for the survival of host organisms (Okamoto & Hirochika 2001).

Gene silencing mechanisms could have developed to protect genomes against transposons. Evidence supporting this hypothesis has come from experiments using *D. melanogaster* and *C.elegans*. In *D. melanogaster*, the normally high transposition rate of the I-element transposon was repressed by the introduction of multiple transgenes with homology to the transposon. The repression appeared to be mediated by an RNA species in a manner very similar to the co-suppression of endogenous genes in plants (Birchler *et al.* 1999). Many *C.elegans* gene silencing mutants also show increased mobilisation of transposons compared to wild type worms (Ketting *et al.* 1999, Tabara *et al.* 1999).

Evidence indicates that dsRNA, in co-operation with proteins, can induce a repressed chromatin state. These findings suggest that defence against transposons may operate at two levels. On one level, any transcribed retrotransposon RNA could be degraded via PTGS, thus preventing the insertion of additional copies into the genome. Secondly, transposon-derived dsRNA could mediate chromatin remodelling, resulting in transcriptional repression of previously inserted copies of the transposon (Birchler *et al.* 1999, Allshire 2002).

1.4 Post-transcriptional gene silencing (PTGS)

1.4.1 Initiation

Several studies have shown that the integration of multiple transgenes in an inverted repeat orientation often results in transgene silencing and co-suppression of homologous endogenous genes (Vaucheret *et al.* 1995, Stam *et al.* 1998). It has been suggested that transcriptional read-through of such transgene loci could yield dsRNA, which in turn could be recognised as aberrant and targeted for degradation (reviewed in Baulcombe 1996 and Meins 2000). However, similar silencing phenomena were observed in plants containing a single transgene copy (Elmayan & Vaucheret 1996, Jorgensen *et al.* 1996, Cluster *et al.* 1996). In these cases, silencing may have been initiated when the level of transcript exceeded some threshold level (Baulcombe 1996). This hypothesis was supported by the observation that the spontaneous silencing of nitrate reductase in transgenic plants is delayed by environmental conditions that reduce the expression these genes (Dorlhac de Borne *et al.* 1994, Palauqui *et al.* 1996). However, the threshold model does not account for the observation that gene silencing is not always associated with highly transcribed transgenes (Stam *et al.* 1997). Moreover, the threshold model would predict a return to the endogenous level of expression, rather then a complete silencing of transgenes as well as endogenous genes. Another possibility is that some of the accumulated transcript contains structural features that make it a target for amplification by an RNA-dependent RNA polymerase (RdRP), an enzyme that converts single-stranded RNA into dsRNA. Alternatively, the transcripts themselves may contain secondary structures, including double stranded loops, which could be a target for PTGS-mediated degradation. Interestingly, transcripts of frequently co-suppressed genes such

as chalcone synthase and nitrate reductase are predicted to form such structures (Metzlaff *et al.* 1997, Berthome *et al.* 2000).

Additional evidence supporting the role of dsRNA as the trigger of PTGS comes from the observation that plant viruses can induce gene silencing. The vast majority of plant viruses have a single-strand RNA (ssRNA) genome that produces double-stranded RNA (dsRNA) molecules during replication. Current evidence suggests that dsRNA acts as a trigger for the plant defence mechanism, which destroys both the replicating virus and its single-stranded genome (reviewed by Achard *et al.* 2004). However, viruses with DNA genomes can also trigger gene silencing (Covey *et al.* 1997, Kjemtrup *et al.* 1998). It is possible that the bidirectional transcription strategy (Gutierrez 1999) used by these viruses results in the formation of RNA-RNA duplexes that, in turn, trigger gene silencing (Fig. 1.2).

The requirement for RNA-RNA duplexes in the initiation stage of gene silencing is further supported by the demonstration that complete silencing of a variety of transgenes, endogenous genes and viral genes can be induced by constructs deliberately designed to produce dsRNA (Waterhouse *et al.* 1998, Smith *et al.* 2000, Baulcombe 2004). Moreover, in diverse experimental models including *Drosophila*, *C. elegans*, the mouse embryo and trypanosomes, PTGS can be efficiently triggered by the introduction of pre-synthesised dsRNA (Montgomery *et al.* 1998, Montgomery & Fire 1998, Ngo *et al.* 1998, Wianny & Zernicka-Goetz *et al.* 2000).

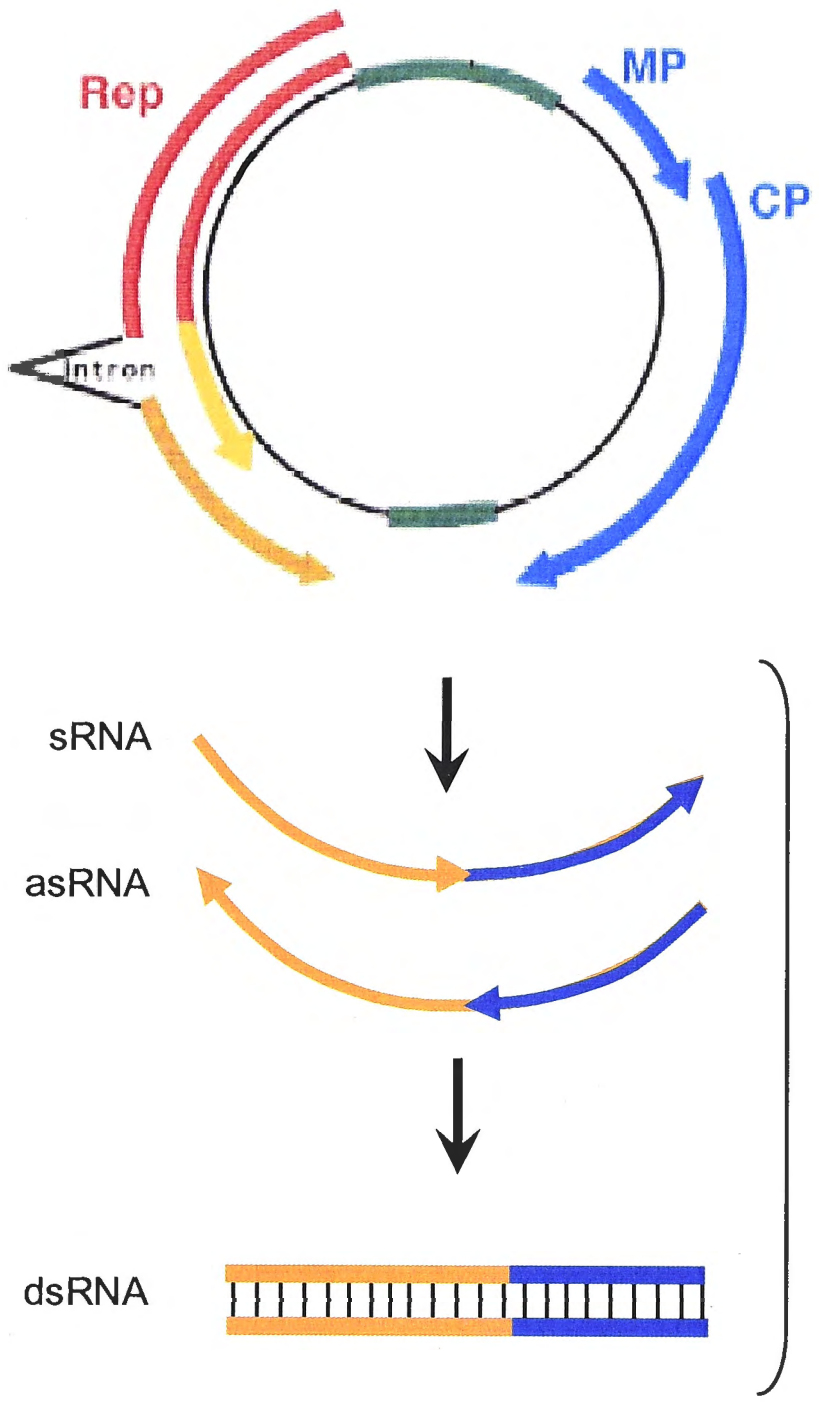


Figure 1.2: Genomic organisation of a geminivirus and a possible result of bidirectional transcription. (adapted from Gutierrez 1999)
Arrows indicate viral proteins and their direction of transcription.

Abbreviations: Rep: replication protein; MP: movement protein; CP: capsid protein; sRNA: sense RNA; asRNA: anti-sense RNA; dsRNA: double stranded RNA.

1.4.2 Mechanism

Several different experimental systems, including plants, *Drosophila* and *C. elegans*, have been used to study the molecular mechanism behind PTGS. The first clues came from plants, with the identification of small interfering 22-25bp RNA (siRNA) species that showed homology to the gene sequences subject to PTGS (Hamilton & Baulcombe 1999). Such siRNA species have been detected in both transgene and virus-induced cases of PTGS and are likely to be the degradation products of the target RNA molecules. Subsequently, the establishment of *in vitro* assay systems has helped identify other components involved in PTGS (Tuschl *et al.* 1999). Research in *Drosophila* identified a dsRNA specific endonuclease called 'Dicer', composed of two RNase III domains and a helicase domain (Bernstein *et al.* 2001). RNase III domains are likely to be responsible for cleavage of dsRNA at 22bp intervals whereas the helicase domain could unwind the dsRNA species and release siRNAs (Fig. 1.3, A) (Bernstein *et al.* 2001). Recent studies have shown that plants and *Drosophila* have more then one type of Dicer (reviewed in Schauer *et al.* 2002). Particular types of Dicer proteins are essential for siRNA-mediated silencing while the others are required for miRNA-mediated control of gene expression during growth and development (Finnegan *et al.* 2003, Lee *et al.* 2004, Pham *et al.* 2004, Tijsterman & Plasterk 2004).

Following Dicer-mediated cleavage of dsRNA, siRNAs are integrated into an RNA-induced silencing complex (RISC) (Fig. 1.3, B) (Hammond *et al.* 2000, Yang *et al.* 2000). It is possible that Dicer directly transfers siRNAs to RISC in a transient interaction. RISC-associated siRNAs function as sequence specificity determinants, guiding the complex to the target ssRNAs, which are subsequently cleaved. The cleavage site has been located to the middle of the siRNA/target RNA duplex region.

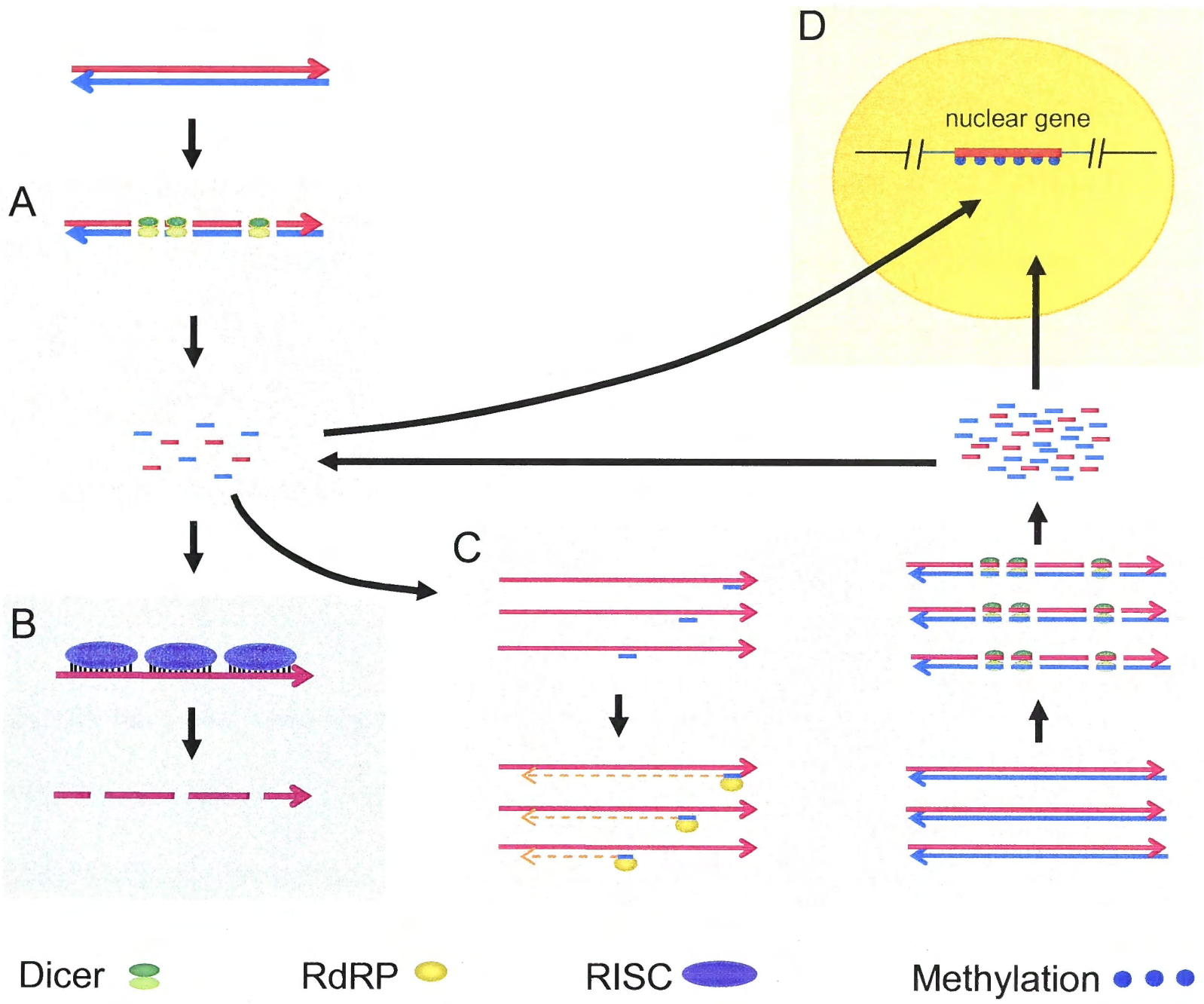


Figure 1.3: **A model for PTGS mechanism.**

A. PTGS is triggered by a small amount of dsRNA that can be of endogenous or viral origin. Dicer mediates cleavage of dsRNA into siRNA species.

B. siRNAs annealed to a homologous mRNA are used as primers by RdRP which amplifies the target mRNA thereby generating more dsRNA.

C. Newly synthesised dsRNA is cleaved by Dicer resulting in large quantities of siRNAs.

D. siRNAs mediate methylation of homologous DNA sequences.

Any mismatches between the siRNA and the target RNA in this region prevent the cleavage (Elbashir *et al.* 2001a, Elbashir *et al.* 2001b, Elbashir *et al.* 2001c).

In addition to being a component of the RISC complex, siRNAs could be used to prime the amplification of mRNAs containing homologous sequences, thereby generating more dsRNA (Fig. 1.3, C) (Lipardi *et al.* 2001, Sijen *et al.* 2001a). This step is likely to involve an RNA-dependant RNA polymerase (RdRP), such as those identified in *Arabidopsis* and *C. elegans* PTGS mutants (Dalmay *et al.* 2000, Smardon *et al.* 2000, Sijen *et al.* 2001a). This amplification of the inducing dsRNA may account for the observation that even very small amounts of dsRNA are able to initiate widespread silencing (Fire *et al.* 1998).

siRNAs have also been shown to direct methylation of homologous DNA sequences (Fig. 1.3, D). However, the role of siRNA-directed methylation is not clear. In a recent study, Rodman *et al.* (2002) analysed the effect of 5-azacytidine (5-Aza-C), an inhibitor of methylation, on virus induced PTGS. The authors found that 5-Aza-C treatment does not prevent the onset of silencing. However, 5-Aza-C treated plants displayed patchy, localised silencing instead of uniform, whole-plant silencing observed in the controls. These findings suggest that methylation is not necessary for the initiation of silencing, but may play a role in its maintenance (Morel *et al.* 2000, Rodman *et al.* 2002).

1.4.3 Proteins involved in PTGS

Biochemical and genetic studies conducted in diverse organisms have identified several proteins required for gene silencing. These components of the gene silencing

machinery tend to be highly conserved indicating that gene silencing is an ancient and essential cellular process.

As described above, the Dicer protein was first identified from biochemical studies in *Drosophila* as the protein involved in degradation of dsRNA. Dicer homologues have since been identified in *C.elegans*, *Arabidopsis thaliana*, *Oriza sativa*, mammals and *Schizosaccharomyces pombe* (Bernstein *et al.* 2001, Golden *et al.* 2002, Schauer *et al.* 2002). In some organisms more than one Dicer-like protein has been identified (Fig. 1.4). Close analysis revealed that most proteins belonging to the Dicer family share the following predicted structure: an N-terminal RNA helicase, a DUF283 domain, a PAZ domain, two RNaseIII motifs and at least one dsRNA bindig domain (Fig.1.4). Some members of Dicer family lack one or more of these domains, while others contain additional motifs such as a nuclear localisation signal or a zinc finger motif.

Of the four Dicer proteins in *A. thaliana*, Dicer-like 1 is best characterised. A number of Dicer-like 1 mutants were the subject of studies investigating seed and flower development. Mapping of these mutations revealed that they affected different Dicer domains but all the mutants displayed severe developmental abnormalities, suggesting that Dicer proteins play an important role in the control of gene expression during the all stages of development (Jacobsen 1999, Golden *et al.* 2002, reviewed in Schauer *et al.* 2002). It is now apparent that mutations in Dicer-like 1 result in developmental abnormalities because Dicer-like 1 is required for the miRNA-mediated regulation of gene expression (Finnegan *et al.* 2003). miRNAs are a special class of small RNAs produced by Dicer-mediated cleavage of complex precursor molecules (reviewed in Mallory & Vaucheret 2004). Plant miRNAs are thought to regulate the expression of

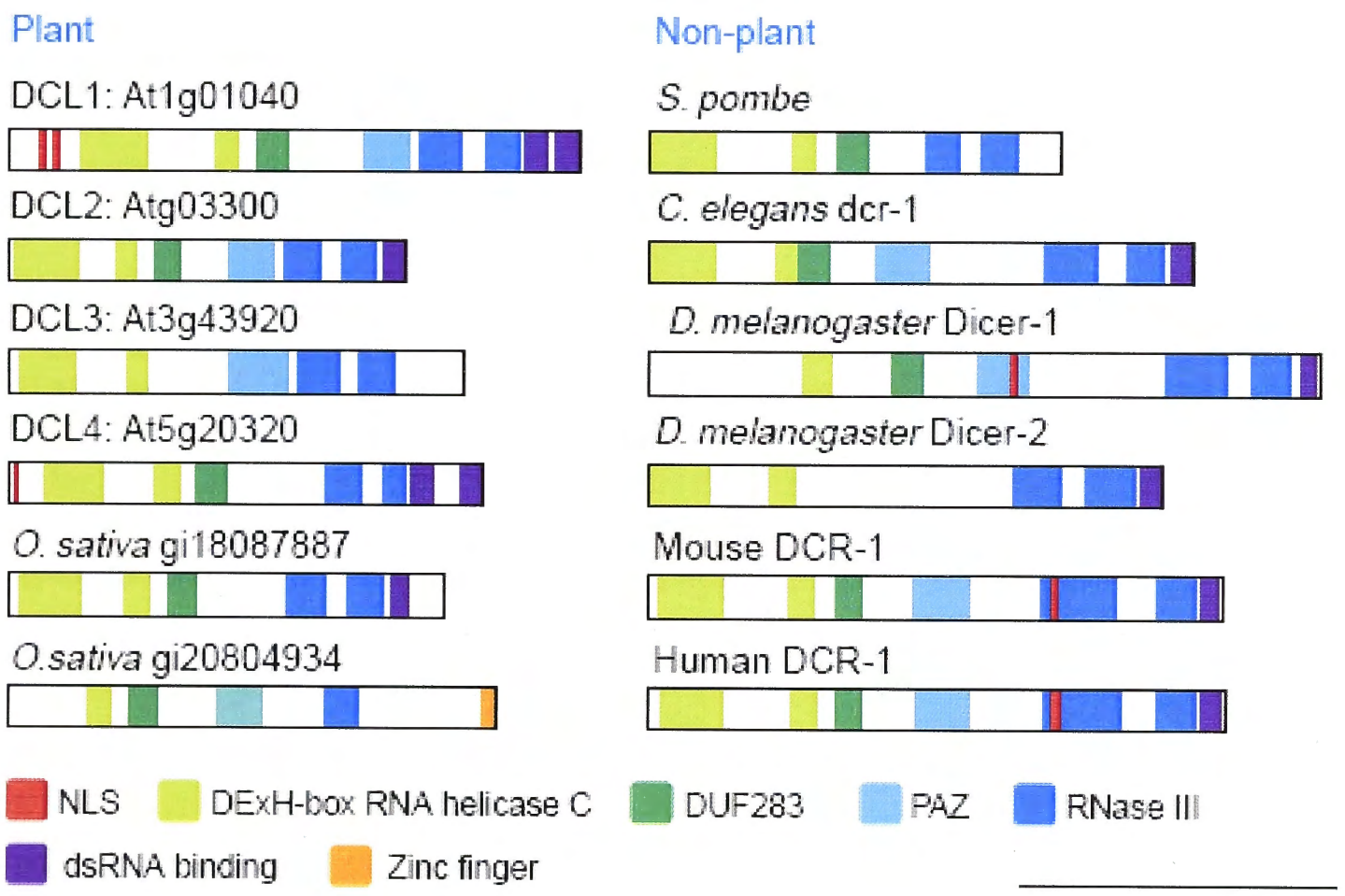


Figure 1. 4: Dicer family members and their predicted domain structure. (Schauer *et al.* 2002)

Abbreviations: NLS, nuclear localisation signal; DUF283, a domain of unknown function; dsRNA binding, double-stranded RNA binding domain; RNase III, ribonuclease III; PAZ, Piwi/Argonaute/Zwille domain.

many transcription factors and other genes by either mediating cleavage or by preventing the translation of their transcripts (Rhoades *et al.* 2002, reviewed in Bartel & Bartel 2003).

Dicer-like 2 appears to be involved in anti-viral defence. Mutations in Dicer-like 2 increase plant susceptibility to viral infection and delay the accumulation of siRNAs derived from the invading virus (Xie *et al.* 2004). Dicer-like 3 is involved in the biogenesis of siRNAs derived from repetitive endogenous sequences, such as SINE retro-elements (Xie *et al.* 2004). Furthermore, plants carrying mutations in Dicer-like 3 exhibit reduced methylation of endogenous loci that are heavily methylated in wild type plants (Chan *et al.* 2004). The precise role of Dicer-like-4 has not yet been determined but recent observations indicate that Dicer-like 4 may be a nuclear protein involved in the production of siRNAs (Papp *et al.* 2003).

Mutations in Argonaute proteins have similar phenotypes to mutations in Dicer-like proteins indicating that these two protein groups act in the same genetic pathway (Lynn *et al.* 1999, Grishok *et al.* 2001). Mutant screens in diverse organisms have shown that members of the Argonaute family play important roles in silencing pathways and in early development. Silencing impaired mutants in *A. thaliana* (AGO-1 and AGO-4), *Neurospora* (QDE-2) and *C.elegans* (RDE-1) all encode Argonaute–like proteins (Tabara *et al.* 1999, Catalanotto *et al.* 2000, Fagard *et al.* 2000, Zilberman *et al.* 2003). In addition, biochemical studies have identified *Drosophila* (dAgo-2) and mammalian (eIF2C2) Argonaute family members as components of the RISC complex (Hammond *et al.* 2001, Hutvagner & Zamore 2002, Martinez *et al.* 2002).

Argonaute proteins are ~100 kDa highly basic proteins composed of two common domains, namely PAZ and PIWI domains (Carmell *et al.* 2002). Recent studies have revealed that the PAZ domain binds siRNAs that guide RISC to the homologous target sequence (Lingel *et al.* 2003, Yan *et al.* 2003, Lingel *et al.* 2004). Once the complex is positioned on the target sequence the PIWI domain cleaves the target RNA (Liu *et al.* 2004, Song *et al.* 2004).

Additional recently identified, members of RISC are the *Drosophila* fragile X related protein (dFXR) and the vasa intronic gene protein (VIG) (Caudy *et al.* 2002, Ishizuka *et al.* 2002). Both of these proteins contain an RGG box domain that is predicted to be involved in RNA binding. Confirmation that these proteins interact with RNA came from biochemical studies which found that dFXR and VIG immunoprecipitates contain ~21nt siRNAs. In addition, these studies found that the proteins believed to be components of RISC, dAgo-2, dFXR and VIG, immunoprecipitate together. These protein precipitates are resistant to RNase-A treatment suggesting that RISC proteins interact with each other, rather then being assembled on a common RNA molecule (Caudy *et al.* 2002).

One of the striking features of PTGS is that very small amounts of dsRNA can inactivate a continuously transcribed target mRNA. The inactivation persists for long periods of time, through cell division and, in case of *C.elegans,* can even be inherited by subsequent generations (Timmons *et al.* 2001). Such potency and the self-sustaining nature of PTGS are likely to be sustained by amplification of the original dsRNA trigger. This hypothesis is supported by the identification of silencing

impaired mutants in *Neurospora* (QDE-1), *C.elegans* (EGO-1 and RRF-1) and *A. thaliana* (SGS2 / SDE-1) with homology to a tomato RNA dependant polymerase (RdRP) (Schiebel *et al.* 1998, Cogoni & Macino 1999, Dalmay *et al.* 2000, Mourrain *et al.* 2000, Smardon *et al.* 2000). Although RdRP homologues have not been found in the *Drosophila* genome, RdRP activity has been identified in its embryo extracts (Lipardi *et al.* 2001). Biochemical studies using *Drosophila* embryo extracts and *C.elegans* demonstrated that siRNAs are used as primers for amplification of target mRNA and the subsequent generation of additional dsRNA (Fig. 1.3) (Lipardi *et al.* 2001, Sijen *et al.* 2001a). Furthermore, distinct specialised RdRPs have been identified in *C.elegans*, where EGO-1 operates in germline cells, while RRF-1 mediates silencing in somatic cells (Smardon *et al.* 2000, Sijen *et al.* 2001a). Analysis of RRF-1 mutants revealed that degradation of trigger dsRNA occurred in these organisms, but did not cause a significant silencing effect (Sijen *et al.* 2001a). This finding suggests that PTGS is a two-step process, initiated by Dicer-mediated degradation of trigger dsRNA into siRNAs and sustained by RdRP-mediated amplification.

Recent studies have indicated that proteins involved in DNA methylation may also be required for PTGS. In plants, cytosine methylation is carried out by three classes of methyltransferases: MET, DRM (domain re-arranged methyltransferases) and CMT (chromomethylases) (Tariq & Paszkowski 2004). The main function of the MET group of methyltransferases appears to be maintenance of CG methylation. The second group of methyltransferases, DRMs, are responsible for de novo methylation of CG, CnG and non-symmetric cytosines while CMTs appear to be involved in maintenance of non-symmetric cytosine methylation (reviewed in Tariq & Paszkowski 2004). Additional proteins, for example DDM1, a member of SNF2/SWI2 family of

chromatin remodelling proteins, are also involved in methylation of plant DNA (reviewed in Finnegan & Kovac 2000).

Mutations in MET1 methyltransferase result in reduced levels of DNA methylation and impaired maintenance of PTGS in developing tissues (Finnegan & Kovac 2000, Morel & Vaucheret 2000). Similarly, *ddm1* mutations cause rapid hypomethylation of repetitive sequences and reduce the efficiency of PTGS in early development (Jeddeloh *et al.* 1999, Finnegan & Kovac 2000, Morel *et al.* 2000).

1.5 Transcriptional gene silencing (TGS)

1.5.1 Initiation

In most cases, TGS is initiated by loci containing multiple transgene copies and then imposed onto other loci that share DNA sequence identity in promoter regions (Vaucheret 1993, Park *et al.* 1996). A hallmark of the silencing process is cytosine methylation, which is first autonomously acquired by the TGS-inducing locus and then imposed onto homologous loci (Vaucheret 1993, Matzke *et al.* 1994, Mette *et al.* 1999). A similar phenomenon has been described in the filamentous fungus *Ascobolus immerses*, where the DNA-DNA pairing triggers *de novo* methylation of all cytosine residues in duplicated sequences (Rossignol & Faugeron 1994, Colot & Rossignol 1999). Based on this observation, it was initially proposed that DNA-DNA interactions could be involved in establishment of TGS and the associated methylation. This view was challenged by Wassenegger *et al.* (1994) who demonstrated that, in transgenic plants, a replicating viroid RNA genome can direct methylation of homologous sequences integrated into nuclear DNA. Even though this study showed that RNA can direct methylation of homologous DNA, it was still not

clear whether RNA derived from promoter sequences could mediate establishment of TGS.

To test whether transcription of a promoter sequence could induce silencing of un-linked genes driven by the same promoter, Mette *et al.* (1999) generated a construct consisting of the nopaline synthase promoter (NOSpro) sequence under control of the cauliflower mosaic virus 35S promoter (35Spro). The construct was introduced into the genome of a plant that already contained an active NOSpro-neomycin phosphotransferase gene (NOSPro-*nptII*). Most transformants produced a full-length NOSPro RNA which did not induce silencing of NOSPro-*nptII*. However, NOSPro-*nptII* was silenced in a plant containing two incomplete copies of the 35S-NOSPro construct integrated as an inverted repeat. The authors proposed that read through transcription of this repeat could produce double stranded NOSPro RNA with a hairpin structure. To test whether such RNA could mediate TGS the authors introduced inverted DNA repeats expressing NOSPro RNA hairpins into plants containing an active NOS-*nptII* gene (Mette *et al.* 2000). The expression of the NOSPro hairpin RNA resulted in sequence–specific DNA methylation and efficient silencing of the previously active NOS-*nptII* gene. Furthermore, NOSPro hairpin RNA was degraded into small RNA species (~23bp) similar to those observed in plants exhibiting PTGS (Hamilton & Baulcombe 1999, Mette *et al.* 2000). The crucial role of dsRNA in the initiation of TGS was confirmed by a subsequent study demonstrating that RNA viruses carrying a portion of the 35S promoter are able to induce TGS of 35S-green fluorescent protein (35S-GFP) gene constructs (Jones *et al.* 2001).

1.5.2 Mechanism

PTGS and TGS share some important common features: both are sequence specific, both are mediated by dsRNAs and both are associated with accumulation of small interfering RNAs (siRNAs) which are thought to be involved in the degradation of homologous mRNA and the methylation of homologous DNA sequences (Mette *et al.* 1999, Hamilton & Baulcombe 1999, Mette *et al.* 2000, Wang & Waterhouse 2000, Jones *et al.* 2001, Sijen *et al.* 2001b). These observations led to a proposal that PTGS and TGS are mechanistically related (Sijen *et al.* 2001b).

A recent study conducted in *Schizosaccharomyces pombe* has demonstrated that the cellular machinery involved in PTGS is also required for the establishment of transcriptional repression at centromeric regions (Volpe *et al.* 2002). Deletion of the genes encoding *S.pombe* homologues of argonaute, Dicer, and RNA-dependant RNA polymerase (RdRP) proteins resulted in the aberrant accumulation of double stranded transcripts from centromeric repeats and transcriptional de-repression of transgenes integrated at the centromere. The authors proposed that the formation and maintenance of heterochromatin could be mediated by dsRNA arising from centromeric repeats. Such transcripts would be processed into siRNAs via a PTGS-like mechanism and then complexed with histone-modifying enzymes to direct their activity to homologous DNA sequences. Loss of histone H3 lysine-9 methylation in argonaute, Dicer and RdRP mutants suggests that one such complex could be formed between centromere-derived siRNAs and a histone methyltransferase (Volpe *et al.* 2002, Verdel *et al.* 2004).

ATP-dependent chromatin remodelling complexes also appear to be involved in dsRNA-mediated transcriptional repression. A lesion in DDM1 (Decrease in DNA Methylation), a member of SWI2/SNF2 family of chromatin remodelling proteins, results in reduced levels of DNA methylation, high levels of transposon reactivation, accumulation of developmental defects and release of TGS in *A. thaliana* plants (Morel *et al.* 2000, Stevenson & Jarvis 2003). Furthermore, in *ddm1* mutants, methylation of histone 3 lysine 9 (H3-K9) associated with inactive chromatin appears to be lost (Gendrel *et al.* 2002). These observations suggest that the function of DDM1 may be to enable access of histone methyltransferases to chromatin.

Recent evidence suggests that methylation of histone 3 lysine 9 results in the recruitment of DNA methyltransferases and other heterochromatin associated proteins. In *Neurospora crassa* the loss of the histone H3 methyltransferase results in the complete loss of DNA methylation (Tamaru & Selker 2001). Similarly, the loss of function of a plant histone methyltransferase KRYPTONITE (KYP) results in reduced DNA methylation at sites usually targeted by the maintenance methyltransferase CMT3 (Jackson *et al.* 2002). These observations suggest that histone methylation can direct activity of DNA methyltransferases.

The link between DNA methylation, chromatin remodelling and the regulation of gene expression has been demonstrated in animals. Proteins that contain methyl-cytosine binding domains (MBD), such as methyl-CpG-binding protein (MeCP2), bind to methylated DNA and recruit histone deacetylases (HDACs) (Nan *et al.* 1996, Nan 1998). Histone de-acetylation results in the establishment of a compact chromatin state which prevents transcriptional initiation. Homologues of MBDs have been found

in *A.thaliana* and maize suggesting that DNA methylation could direct HDAC activity in plants (Fransz & de Jong 2002). Deacetylation of histone tails, followed by methylation of these tails could be the final steps in the establishment of the repressed chromatin state associated with TGS.

Taken together, these findings suggest that the cooperative action of a number of proteins is required for the establishment and maintenance of TGS. In particular, there appears to be cross talk between DNA and histone methyltransferases enzymes that play a major role in this process. Fig. 1.5 outlines a model of TGS mechanism based on this premise.

1.5.3 Proteins involved in TGS

Dicer, Argonaute proteins and DNA methyltransferases are involved in both PTGS and TGS and have been reviewed in Section 1.4.3. Additional proteins involved in the establishment of the repressed chromatin state that is characteristic of TGS are discussed below. Mutant screens have confirmed the involvement of some of these proteins in TGS, while the involvement of others is only hypothesised because of their association with repressed chromatin states.

Chromatin remodelling complexes of the SWI/SNF family appear to play an important role in the establishment of TGS. These proteins are characterised by the presence of a bromo-domain and a catalytic ATPase subunit (Fig. 1.6) (Narlikar *et al.* 2002). A recent model proposes that the bromo-domain could target these proteins to chromatin, while hydrolysis of ATP by the ATPase subunit could provide energy to weaken histone-DNA interactions. Consequently, histones and DNA would become more

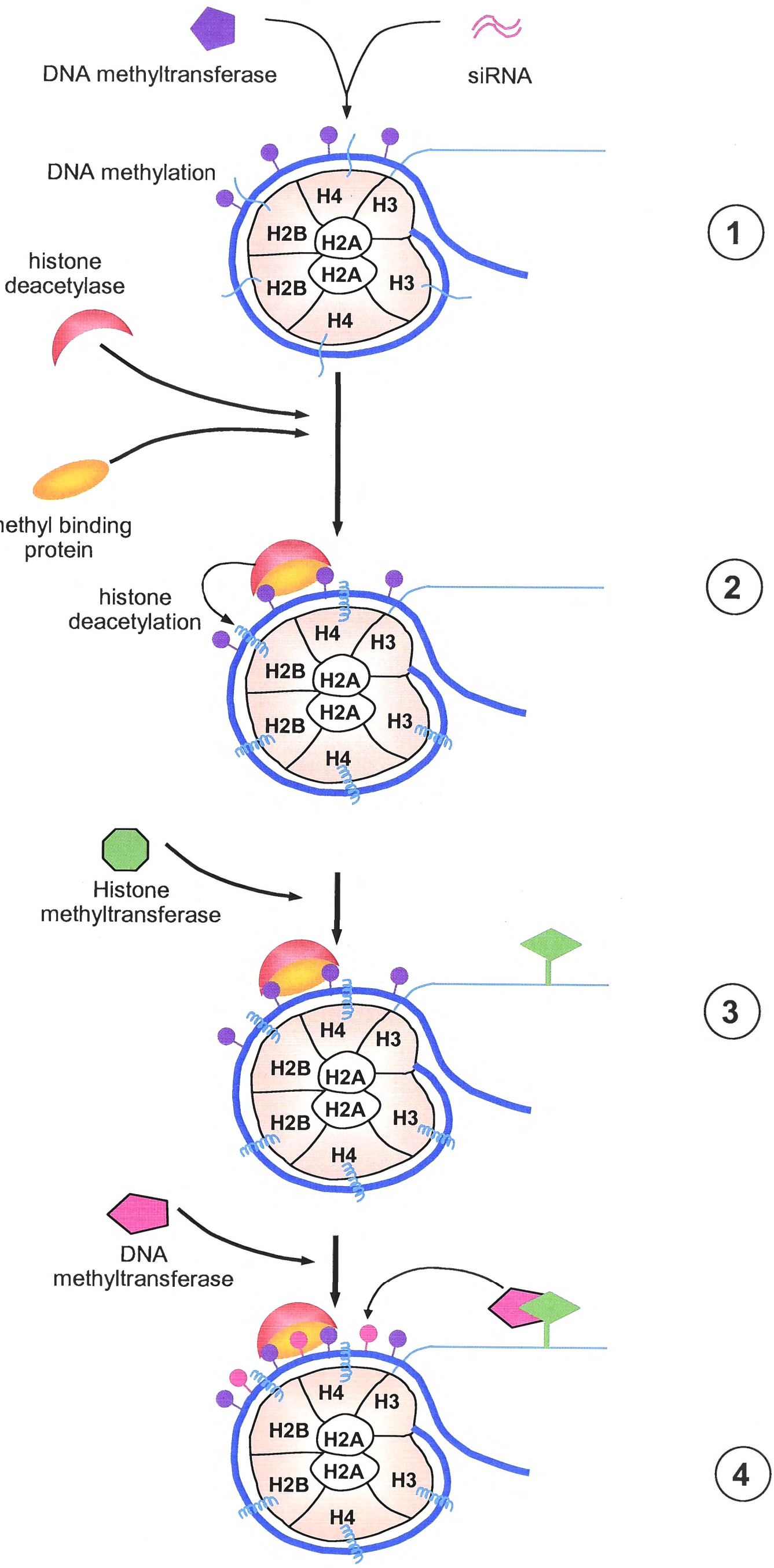
Figure 1.5: A model for the establishment of TGS

1. *de novo* methyltransferase activity is directed by siRNAs.

2. Methylated DNA is bound by methyl binding proteins. These proteins may recruit histone deacetylases.

3. Histone methyltransfereases are recruited and H3K9 is methylated.

4. H3K9 methylation initiates the recruitment of additional DNA methyltransferases.



inactive chromatin state

accessible to modifying proteins such as histone and DNA methyltransferases (Gavin *et al.* 2001). This model is supported by the observation that mutations in the *Arabidopsis* SWI/SNF family member, DDM1, result in loss of histone H3-K9 and DNA methylation (Jeddeloh *et al.* 1999, Gendrel *et al.* 2002). Furthermore, mutations in a recently described member of the SWI/SNF family, DRD1, result in loss of RNA-directed DNA methylation (Kanno *et al.* 2004). Loss of methylation is specific to CnG and non-symmetric cytosines suggesting that DRD may interact with *de novo* DNA methyltransferases (Kanno *et al.* 2004).

The Morpheus Molecule (MOM) is another *Arabidopsis* protein with homology to the SWI/SNF family involved in TGS. The predicted product of the MOM gene is a 2001-amino acid nuclear protein with an ATPase domain and a putative helicase region (Amedeo *et al.* 2000). The helicase region appears to encode only one domain of the helicase unit, which is normally comprised of two domains, indicating that MOM could dimerise with a protein which carries the second domain (Amedeo *et al.* 2000, Chandler & Jorgensen 2000). Mutations in the MOM gene result in the release of TGS, but do not have an effect on DNA methylation patterns or plant phenotype (Amedeo *et al.* 2000). Based on these observations two alternative modes of action of MOM could be envisaged. One possibility is that MOM recognises DNA methylation as a silencing signal and mediates downstream events resulting in TGS. Alternatively, MOM could be involved in the silencing pathway independent of DNA methylation. The latter hypothesis is supported by the observation that *ddm/mom* mutations have additive effects leading to severe chromosomal and developmental abnormalities (Scheid *et al.* 2002).

Histone methyl-transferases (HMTases) are a highly conserved family of proteins. Members of this family have been identified in *Drosophila* (SU(VAR)3-9), *S.pombe* (Clr4), mammals (SUV39H1 and H2), *Neurospora crassa* (DIM-5) and in *Arabidopsis* (KRYPTONITE) (Rea *et al.* 2000, Tamaru & Selker 2001, Baumbusch *et al.* 2001, Jackson *et al.* 2002, Schotta *et al.* 2003). These proteins tend to be associated with heterochromatin and share several domains associated with chromatin modulation (Fig. 1.6) (Melcher *et al.* 2000, Nakayama *et al.* 2001). The SET domain and the two flanking cysteine rich domains (Pre-SET and Post-SET) are thought to mediate methylation of H3K9, while the N-terminus appears to interact with heterochromatin protein 1 (HP1). Evidence suggests that the chromodomain could function as an RNA-interaction module, which raises a possibility that siRNAs target HMTases to homologous DNA sequences (Fujita *et al.* 2003, Fuks *et al.* 2003, Schotta *et al.* 2003). The observation that the chromodomain of *Drosophila* (SU(VAR)3-9) HMTase is essential for its targeting to chromocenter heterochromatin appears to support this hypothesis (Schotta *et al.* 2002).

HP1 is a protein involved in the generation and maintenance of an inactive heterochromatin structure. It was initially identified and characterised in *Drosophila*, however subsequent studies have found HP1 homologues in a large number of organisms including yeast, nematodes, mammals and plants (Eissenberg & Elgin 2000, Baumbusch *et al.* 2001, Gaudin *et al.* 2001). Defining characteristics of the HP1 family are an amino-terminal chromo-domain and a structurally related carboxy-terminal chromo-shadow domain (Fig. 1.6). The HP1 chromo-domain interacts with methyl-H3-K9 while the chromo-shadow domain appears to mediate self-association (Bannister *et al.* 2001, Lachner *et al.* 2001, Gaudin *et al.* 2001, Jackson *et al.* 2002).

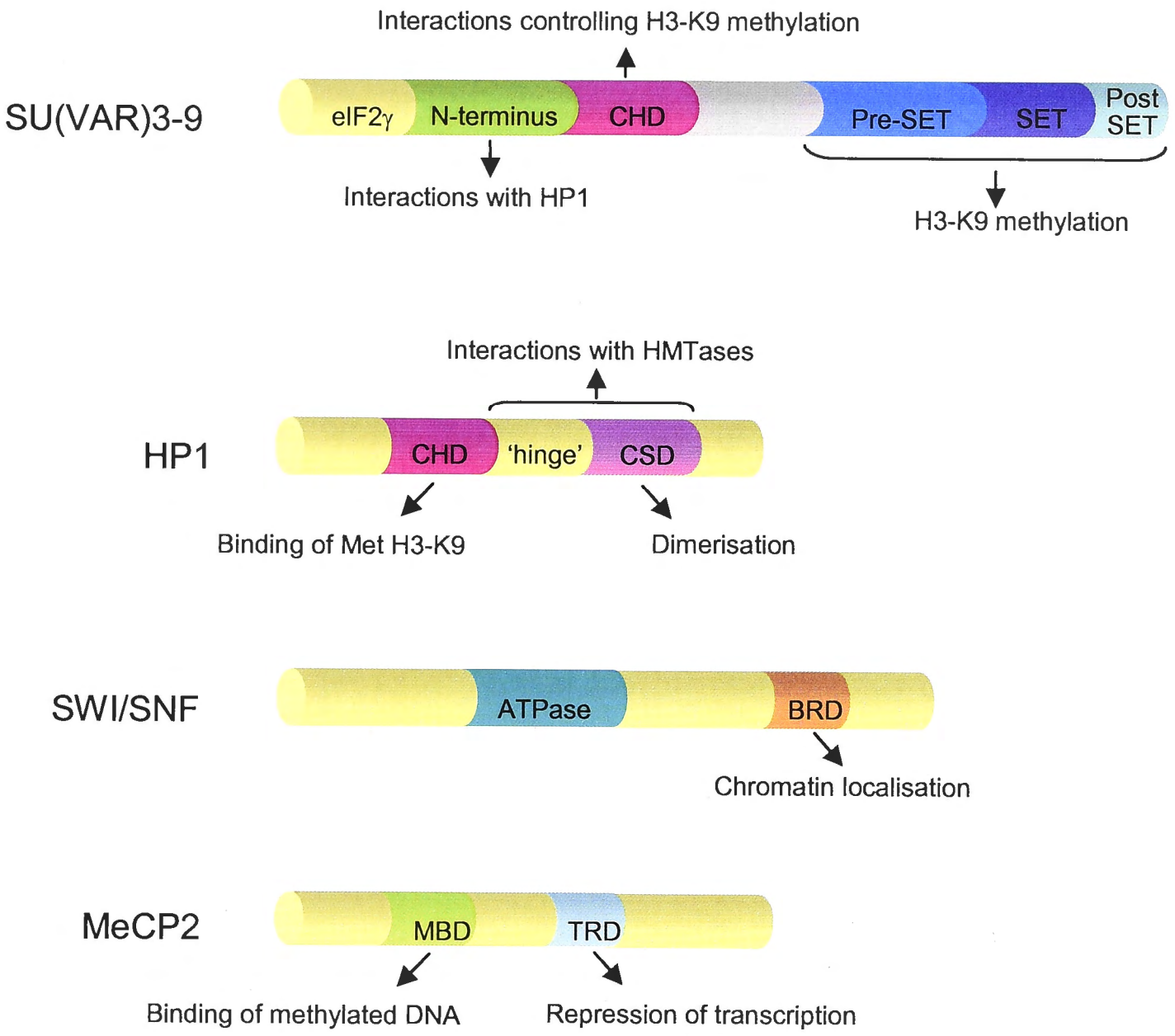


Figure 1.6: Domain structures of selected proteins involved in TGS.

Known domain functions are indicated.

Abbreviations: CHD: chromo-domain; H3-K9: histone 3 lysine 9; CSD: chromo shadow domain; BRD: bromo-domain; MBD: methyl-binding domain; TRD: transcriptional repression domain.

A recently identified *Arabidopsis* homologue of HP1, LHP1 (Like Heterochromatin Protein 1), is involved in the formation of heterochromatin-like repressive complexes and developmental control of gene expression. Mutations in LHP1 lead to de-repression of developmentally regulated genes and alterations in flowering time, leaf development and plant architecture (Gaudin *et al.* 2001, Kotake *et al.* 2003).

Methyl CG binding proteins bind methylated DNA in a sequence non-specific manner and recruit histone deacetylase (HDAC) complexes leading to chromatin compaction and transcriptional repression (Ballestar & Wolffe 2001). The founding member of the methyl-binding protein family is MeCP2 which consists of a methyl CG binding domain (MBD) and a transcriptional repression domain (TRD) (Fig. 1.6) (Wade 2001, Ballestar & Wolffe 2001). Database searches revealed that 12 putative MBD proteins exist in the *A.thaliana* genome. Further biochemical analysis demonstrated that at least three of these candidates bind methylated DNA and precipitate with histone deacetylase activity (Zemach & Grafi 2003).

Three types of histone deacetylases (HDACs) have been identified in plants. Two of these have homologues in other eukaryotes, while the third group of histone deacetylases (HD2 type) is specific to plants (Pandey *et al.* 2002). Antisense-induced downregulation of *Arabidopsis* AtHD1 deacetylase causes developmental defects and reactivation of silenced loci. These observations suggest that histone deacetylation mediates not only the establishment of the repressed chromatin state, but also its maintenance (Wu *et al.* 2000, Tian & Chen 2001). Although histone deacetylation has been associated with the repressed chromatin state in numerous organisms, its role in the mediation of repressed chromatin states is not well understood. One possibility is

that histone deacetylation mediates transcriptional silencing by preventing the assembly of the transcriptional machinery. Alternatively, deacetylated histones could induce the formation of a higher order chromatin structure that is not compatible with transcription (Ng & Bird 2000).

1.6 Systemic spread of gene silencing

When silencing is spontaneously triggered in transgenic plants it is usually confined to a small number of cells. However, silencing can often spread systemically to affect most of the plant tissues (Boerjan *et al.* 1994, Palauqui *et al.* 1996). To date there is limited information about the nature and spread of the signal that causes systemic silencing. It has been suggested that the signal could consist of double stranded RNAs or siRNAs which could be transported either as naked RNA or in a complex with a carrier protein. Information about the transport of the silencing signal is scarce, however, the silencing signal is likely to have similar transport patterns to the well-studied movement of endogenous and viral RNAs. In order to generate a more complete picture of possible transport mechanisms of the silencing signal relevant plant physiology and research on movement of endogenous and viral RNAs will be reviewed.

1.6.1 Plant physiology

The aspects of plant physiology most relevant to the movement of the silencing signal concern plant vasculature and cell-to-cell communication pathways. The plant vascular system is comprised of xylem and phloem conducting elements, which are found juxtaposed in vascular bundles (Fig. 1.7). Xylem functions as a water conduit

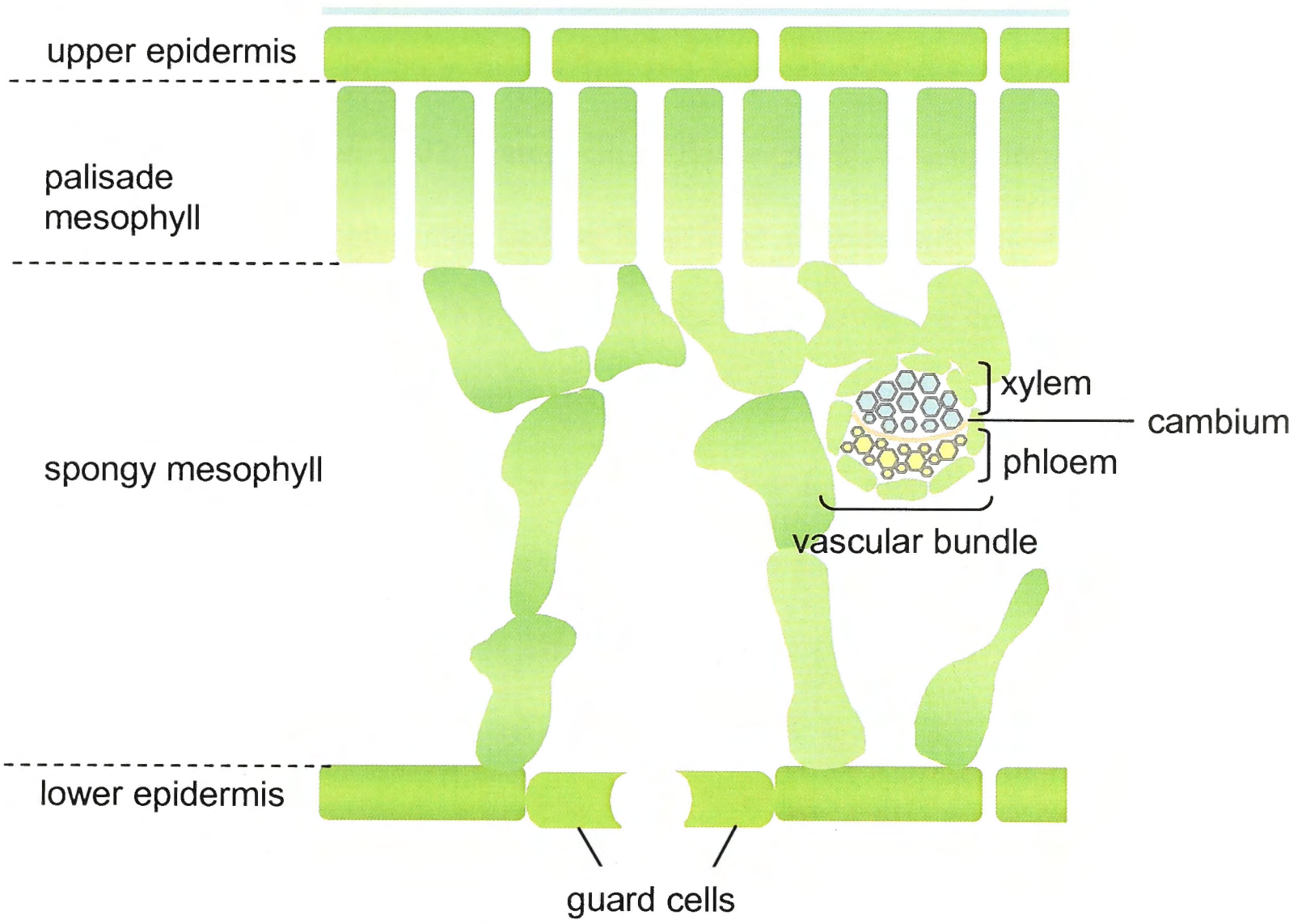


Figure 1.7: Transverse section of a plant leaf.

for the plant, while nutrients, hormones and RNAs are transported in the phloem. Cell-to cell transport is regulated by channels called plasmodesmata.

1.6.1.1 Phloem

Phloem is composed of two types of cells, sieve elements and companion cells, which originate from the same phloem mother cell (Fig. 1.8). As they mature, sieve cells lose nuclei, vacuoles, most of their organelles and the capacity for transcription and translation (Alberts *et al.* 2002, Campbell & Reece 2002). Plasmodesmata linking sieve tube elements become modified to form large plasma membrane-lined sieve plate pores, thereby establishing a highly specialised conduit for the delivery of sugars, hormones, amino acids, proteins and RNA to the whole plant (Fig. 1.8 C). In contrast to sieve elements, companion cells are densely cytoplasmic and exhibit a high rate of cellular activity. Many macromolecules required for the maintenance of sieve elements are imported from companion cells via specialised companion cell – sieve element plasmodesmata (Fig. 1.8 B) (reviewed in Lucas *et al.* 2001).

1.6.1.2 Plasmodesmata

Plasmodesmata establish cytoplasmic continuity between neighbouring cells. This allows intercellular exchange of small molecules such as ions, metabolites and hormones and thereby enhances coordination of biochemical and physiological processes (Alberts *at al.* 2002, Campbell & Reece 2002). Furthermore, recent evidence shows that plasmodesmata are also used to mediate selective cell-to-cell transport of proteins and ribonucleoproteins (reviewed in Lucas *et al.* 2001).

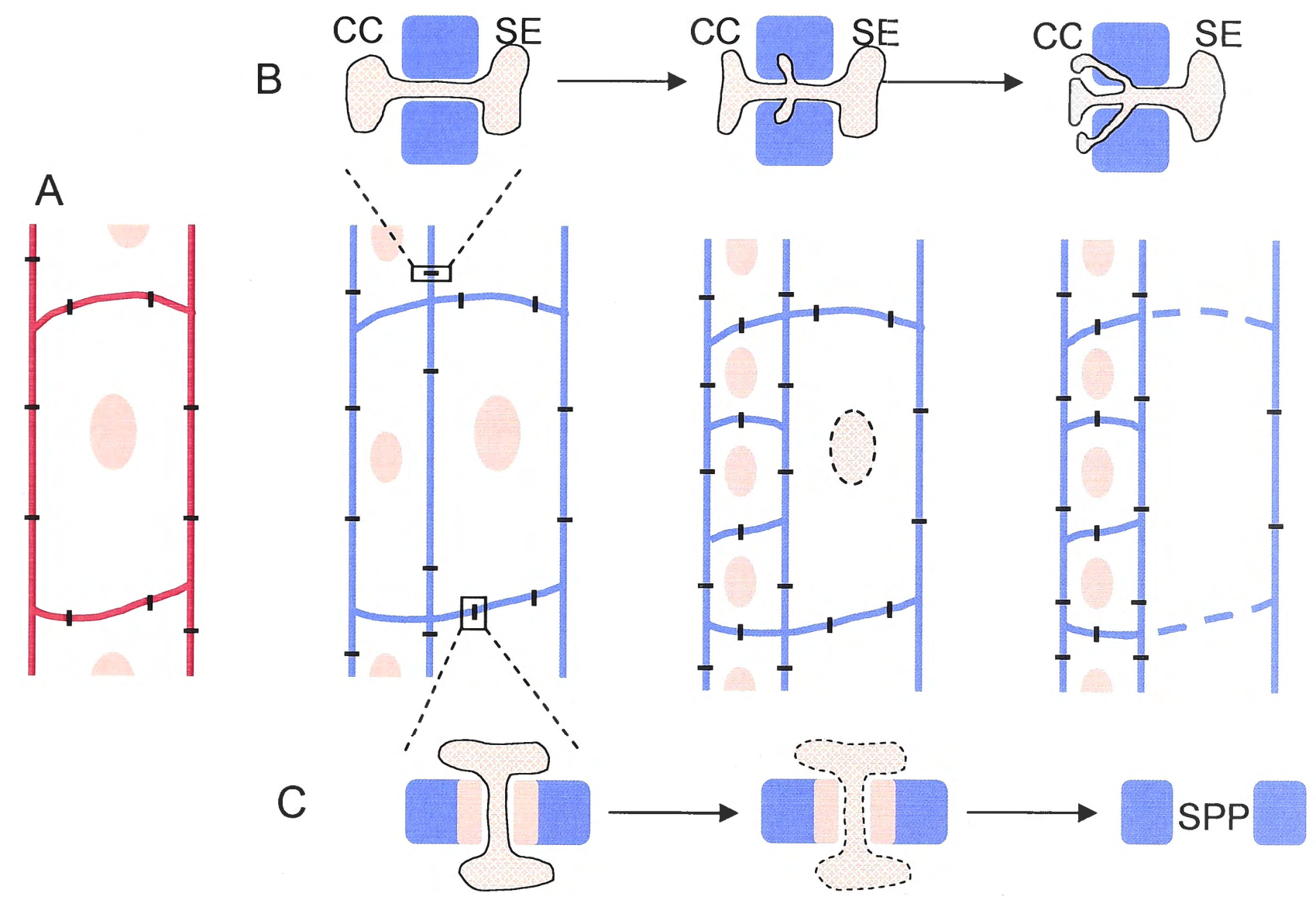


Figure 1.8: Development of the phloem sieve-tube system. (adapted from Lucas *et al.* 2001)

A Schematic representation of phloem development

B Structural modification of plasmodesmata that connect companion cells (CC) to sieve elements (SE)

C Removal of plasmodesmata that connect SE results in formation of sieve pore plates (SPP).

Plasmodesmata have two major components – membranes and spaces (Fig. 1.9). The plasma membrane between adjacent cells represents the outer boundary of plasmodesmata. The centre of plasmodesmata, termed the desmotubule, is made up of cylindrically shaped endoplasmic reticulum that is continuous between neighbouring cells. The space between the desmotubule and the plasma membrane is called the cytoplasmic annulus and is likely to be the major conduit through which molecules pass from cell to cell. Proteins that interconnect the plasma membrane and the desmotubule may act to expand or contract the annulus, thereby regulating transport through plasmodesmata (reviewed in Lucas & Wolf 1993, Zambryski & Crawford 2000). These general plasmodesmal features can vary depending on the location and origin of the plasmodesmata (Table 1.1).

1.6.2 RNA trafficking on the vascular superhighway

1.6.2.1 Endogenous RNAs

RNA trafficking within a plant can be divided into two components: cell-to-cell transport that requires passage through plasmodesmata, and long distance transport via phloem.

A well studied example of the movement of endogenous RNAs involves RNA transport between companion cells (CC) and sieve elements (SE). Kuhn *et al.* (1997) found that mRNA of the sucrose transporter SUT1 in potato plants is transcribed in companion cells, then translocated to adjacent sieve elements and distributed systemically, via the phloem, to its final destination where it is translated (Kuhn *et al.* 1997, Lucas *et al.* 2001). In pumpkin plants, RNA transport is mediated by the CmPp16 protein which binds RNAs in a sequence non-specific fashion facilitating

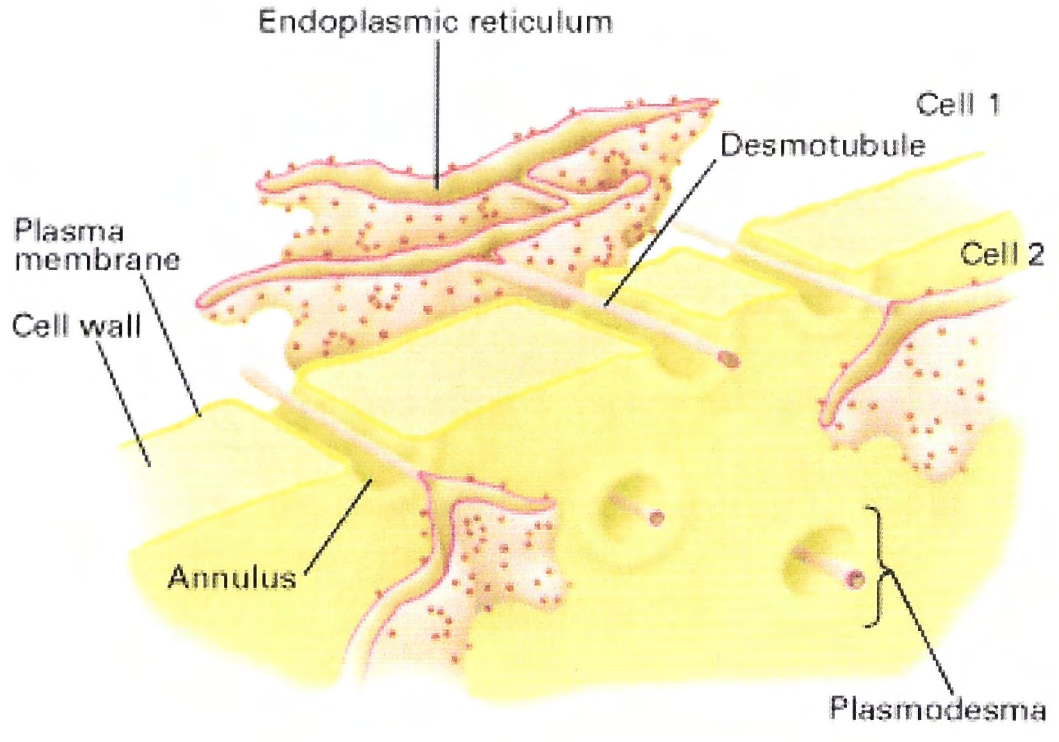


Figure 1.9: Structure of the plasmodesmata. (Lodish *et al.* 2000)

Table 1.1: Characteristics of primary, secondary and CC-SE plasmodesmata.

	Primary plasmodesmata	Secondary plasmodesmata	CC-SE plasmodesmata
Location	mesophyll cells	mesophyll cells and graft junctions	CC-SE junctions
Time of development	during cell division	after cell division	maturation of SE
Structure	single channel	single channel or branched channels	branched on CC side
Size exclusion limit (SEL)	1 kDa	1kDa	up to 40 kDa

their transport from CC to SE by increasing the size exclusion limit (SEL) of plasmodesmata (Xoconostle-Cazares *et al.* 1999, Lucas *et al.* 2001). However, cell-to-cell RNA transport is not confined to CC and SE. For example, the maize homeodomain protein, Knotted 1, binds its own mRNA and transports it between adjacent cell layers within meristem tissue (Lucas *et al.* 1995). Similarly, the *A. thaliana LEAFY* (*LFY*) transcription factor mRNA can be transported through the apical meristem, into the neighbouring cell layers where it acts to rescue a *lfy* mutation (Sessions *et al.* 2000).

A large population (>500) of polyadenylated mRNA molecules has been localised to phloem sap (Ruiz-Medrano *et al.* 1999, Xoconostle-Cazares *et al.* 1999, Kim *et al.* 2001, Lucas *et al.* 2001). Heterografting experiments, where a scion from one species is grafted onto a rootstock of another species, have been instrumental in demonstrating that such RNA species move over long distance through phloem. For example, in heterografts where pumpkin acts as the rootstock and cucumber as the scion, a pumpkin *CmNACP* mRNA is targeted to the cucumber shoot apex where it appears to be involved in apical meristem development (Ruiz-Medrano *et al.* 1999). Transport of such mRNAs through pumpkin phloem appears to be facilitated by the CmPp16 protein which chaperones transport of its own, as well as other, RNAs (Xoconostle-Cazares *et al.* 1999).

Kim *et al.* (2001) elegantly demonstrated that transported RNA species perform regulatory functions at their end destination. The authors performed grafting experiments using two different tomato lines; the *Xa* tomato line carrying a semi-dominant mutation that causes yellow leaves with wild-type morphology, and another

tomato line with the *Mouse ears* (*Me*) mutation causing leaves with extra orders of compounding. Grafting *Xa* scions onto *Me* rootstocks caused distinct phenotypic changes in leaf morphology, resulting in extra compounding of the newly emerging leaves of the *Xa* scion. Examination of the shoot apex under a scanning electron microscope showed that these changes occurred very early in leaf development. The *Me* mutation is caused by a gene fusion termed *PFP-LeT6*, between pyrophosphate-dependent phosphofructokinase (*PFP*) and tomato *KN-1*-like homeobox gene *LeT6*. Transport of this fused transcript can therefore induce the *Me* mutant phenotype in a genetically normal scion. Reciprocal grafting experiments where *Me* scions were grafted onto *Xa* rootstock did not result in a change of phenotype of the *Xa* rootstock. This observation could be explained by unidirectional (from source to sink) transport of change-mediating RNA. Alternatively, rootstock leaves could already be beyond the developmental stage at which the *PFP-LeT6* transcript can induce a change in leaf morphology.

Identification of endogenous RNA species that move between plant tissues suggests that the endogenous mRNAs can serve as long distance systemic signals for plant development and morphogenesis. Interestingly, endogenous transcripts such as *PFP-LeT6* and *CmNACP* are specifically targeted to meristematic tissue, while viruses and gene silencing elicitors are capable of spreading throughout most of the plant, but are often prevented from reaching very young meristematic tissue (Ruiz *et al.* 1998). Thus, plants may have evolved a communication barrier that protects developmentally important tissues from viral infection and gene silencing, and yet remains permeable for specific endogenous mRNAs (reviewed in Haywood *et al.* 2002).

1.6.2.2 Viral genomes

When a mechanically transmitted virus is introduced into a plant, it is usually deposited in a few epidermal cells. The spread of virus from this initial site of infection is a multi-step process. The first requirement is that the infected cells must support viral replication. Secondly, the virus, or its genome, must move from epidermal into mesophyll cells of the initially infected leaf. The third phase involves virus entry into the sieve element network and passive transport over long distances into sink tissues (Cruz *et al.* 1996, Lazarowitz & Beachy 1999). Finally, the virus must exit the vasculature and re-establish infection in tissues distant from the initial site of infection (Cruz *et al.* 1996, Gilbertson & Lucas 1996).

Cell-to-cell movement is an active process mediated by virus-encoded movement proteins and some host factors. Three distinct mechanisms for viral movement have been described. The first involves the production of a single movement protein that can traffic between cells (Heinlein 2002), the second requires the co-operative action of three movement proteins and the coat protein (Cruz *et al.* 1998, Lough *et al.* 2000), while the third mechanism involves transport of virions through tubules that penetrate the walls of adjacent cells (Carrington *et al.* 1996).

One of the best studied examples of movement protein mediated cell-to-cell transport involves the tobacco mosaic virus (TMV) (Heinlein 2002). Its local transport is facilitated by a 30 kDa movement protein (P30). P30 co-operatively binds TMV RNA along its entire length, and forms a nucleoprotein that is much smaller in size than a TMV virion (Citovsky *et al.* 1990, Citovsky *et al.* 1992). This nucleoprotein interacts with microtubules and is targeted to plasmodesmata where P30 mediates an increase in

the size exclusion limit (SEL) of the Pd to 10 kDa (Deom *et al.* 1990, Boyko *et al.* 2000, Boyko *et al.* 2002). Interestingly, the increase in the SEL is limited to the cells at the leading edge of the expanding infection (Oparka *et al.* 1997). As prolonged Pd expansion would be detrimental to normal cellular protein and RNA trafficking, this response represents an adaptation to preserve plant functions while also allowing viral movement.

Cell-to-cell movement of potexviruses is mediated by three proteins known as the triple gene block (TGB) proteins, and a coat protein (Cruz *et al.* 1998). TGB proteins mediate an increase in the SEL of plasmodesmata but are unable to facilitate the movement of virions or viral RNA out of the cell. In contrast, the coat protein does not affect the SEL, however it is necessary for intercellular movement (Cruz *et al.* 1998, Lough *et al.* 2000). The precise role of the coat protein in cell-to-cell movement is not known, however a report that potato virus X virions localise to plasmodesmata of infected cells, suggests that potexviruses may be trafficked in a completely assembled form (Cruz *et al.* 1998).

A strong correlation between competence for virion assembly and cell-to-cell movement indicates that potyviruses could also be trafficked in an assembled form (Dolja *et al.* 1994, Dolja *et al.* 1995). Potyviral CP mediates virion assembly and induces an increase in the plasmodesmal SEL, while the cylindrical inclusion protein (CI) forms structures that direct intracellular translocation of virions (Rodriguez-Cerezo *et al.* 1997). In cells at the edge of an advancing infection front, CI forms cylindrical structures that enclose CP and viral RNA (Roberts *et al.* 1998). These structures localise to plasmodesmal openings suggesting that they are used to position

virions to plasmodesmata where CP induces an increase in the SEL. This in turn results in the translocation of virions into adjacent cells (Roberts *et al.* 1998, Carrington *et al.* 1998).

Viroids represent an interesting alternative to protein-mediated cell-to-cell transport. Viroids are non-translatable, self replicating circular RNAs that can effectively infect plants and spread out of the inoculated cells. The potato spindle tuber viroid has been shown to use plasmodesmata to move from cell to cell. Moreover, it is able to facilitate the movement of an unrelated fused sequence (Ding *et al.* 1997). Such movement may be mediated by a specific sequence or structural motif that either interacts with a plasmodesmal component or an endogenous protein involved in RNA trafficking.

Specialised plasmodesmata located between companion cells and sieve elements control entry to, and exit from the phloem. Although these plasmodesmata have a relatively large SEL, evidence suggests that small size alone is not sufficient for transport (Balachandran *et al.* 1997, Imlau *et al.* 1999). It appears that molecular interactions between viral proteins and CC-SE plasmodesmata determine whether a virus is able to cross the mesophyll-phloem barrier (Itaya *et al.* 2002). Long distance transport of viruses through plant vasculature, which is rich in nucleases, requires viruses to protect their genomes from the hostile environment of the vascular fluid. Thus, most viruses are transported in encapsulated form as virions, while viroids are likely to travel either as naked RNA with high secondary structure or in a complex with endogenous RNA trafficking proteins (Carrington *et al.* 1996).

Most viruses that move from cell-to-cell in a non-encapsulated form require coat proteins for long distance movement. For example, capsid protein mutants of red clover necrotic mosaic virus (RCNMV), tobacco mosaic virus (TMV) and tobacco etch virus (TEV) move from cell to cell efficiently, but exhibit defects in phloem-dependent long-distance transport (Saito *et al.* 1990, Xiong *et al.* 1993, Dolja *et al.* 1994, Dolja *et al.* 1995, Vaewhongs & Lommel 1995). Cucumber mosaic virus (CMV) enters sieve elements as a ribonucleoprotein and a separate coat protein. However, before entering the phloem stream, CMV assembles into virions in a protected environment between the SE plasma membrane and the parietal SE endoplasmic reticulum (Blackman *et al.* 1998). These examples suggest that, for many viruses, encapsidated virions represent a functional long distance movement complex.

Some viruses encode additional proteins that provide functions needed for phloem-dependent, but not cell-to-cell transport. For example, TEV with a mutation in the Helper Component Protease (HC-Pro) moves from cell-to-cell and reaches the sieve elements, however, it cannot establish a systemic infection indicating that the HC-Pro mutation impairs flow through, or exit from, the vascular tissue (Cronin *et al.* 1995, Seron & Haenni 1996, Kasschau *et al.* 1997). Similarly, b2 protein encoded by cucumoviruses and p19 encoded by tombusviruses promote host-specific long-distance movement (Scholthof *et al.* 1993, Ding *et al.* 1995, Scholthof *et al.* 1995a, Scholthof *et al.* 1995b, Desvoyes & Scholthof 2002, Qiu *et al.* 2002, Qu & Morris 2002). It is interesting to note that these proteins have also been implicated in the suppression of gene silencing, suggesting that they may promote systemic viral movement by blocking the host immune response.

Gomez & Pallas (2004) have identified an endogenous protein that forms a ribonucleoprotein complex with hop stunt viroid (HSV) in the phloem of cucumber plants. Although this protein has not yet been characterised, it is possible that it operates in a similar manner to the pumpkin CmPp16 protein that chaperones transport of RNA in a sequence non-specific manner (Xoconostle-Cazares *et al.* 1999). In addition, recent studies indicate that viroid trafficking is tightly regulated by their secondary structure motifs. Mutations that alter the secondary structure of Potato Spindle Tuber Viroid (PSTV) prevent its unloading into floral organs and result in entrapment within the phloem (Zhu *et al.* 2002). These findings suggest that viroids have evolved structural motifs that mimic endogenous plant RNA motifs, so that they can be recognised by cellular factors involved in trafficking.

1.6.2.3 Silencing signals

Propagation of the silencing signal bears a striking resemblance to propagation of viral infection. Like a viral infection, silencing first occurs in a small cluster of cells that are located in a source leaf, and is then propagated up the plant axis in a pattern that reflects the pathway of phloem translocation. (Palauqui & Balzergue 1999, Voinnet *et al.* 1998). In the system described by Voinnet & Baulcombe (1997), silencing of *in planta* expressed GFP can be triggered by agroinfiltration with a GFP encoding construct. Upon infiltration, silencing first occurs in the mesophyll cells of the infiltrated leaf and within 5 days spreads to the stem, then to the upper-most leaves (Ruiz *et al.* 1998, Voinnet *et al.* 1998). Three weeks later, silencing appears around the main vein of older leaves and finally extends to minor veins and the intervenial tissue (Voinnet & Baulcombe 1997, Voinnet *et al.* 1998). An identical pattern of spread is

observed in plant lines in which silencing of nitrate or nitrite reductase transgenes and endogenous genes is triggered spontaneously (Dorlhac de Borne *et al.* 1994, Palauqui *et al.* 1996).

Virions, endogenous RNAs and the silencing signals are all able to move through graft junctions. It has been shown that silencing of nitrate reductase, nitrite reductase, GUS, chitinase and viral genes can be transmitted efficiently from silenced rootstocks to scion tissue expressing the corresponding transgene (Palauqui *et al.* 1997, Palauqui & Vaucheret 1998, Sonoda & Nishiguchi 2000, Crete *et al.* 2001). Furthermore, the transmission of silencing almost always occurred uni-directionally from rootstock to scion. These observations suggest that the silencing signal could move passively through phloem, from mature photosynthetic source leaves of the rootstock to the developing sink leaves of the scion. Alternatively, the silencing signal could reach the mature leaves of the rootstock but these mature leaves may be incapable of implementing silencing.

Findings by Ueki & Citovsky (2001) suggest further similarities between viral movement and the movement of the silencing signal. These authors have found that incubation of plants with non-toxic concentrations of cadmium inhibits the systemic spread of both tobamoviral infection and gene silencing. Both viral infection and silencing occurred in the leaves where they originated but did not spread to the sink leaves. Some viral particles were found in the vasculature, suggesting that cadmium may have interfered with a component of the plasmodesmata guarding re-entry from vasculature into the mesophyll cells (Citovsky *et al.* 1998, Ghoshroy *et al.* 1998). In follow-up studies, Ueki & Citovsky (2002) identified a cadmium-induced glycine-rich

protein (cdiGRP) which was up-regulated in cadmium treated plants. The authors generated transgenic plants over-expressing this protein and demonstrated that systemic spread of tobamoviruses and silencing signals was impaired in these plants. Furthermore, in transgenic plants expressing an antisense *cdiGRP* gene sequence, cadmium treatment did not prevent systemic spread of tobamovirus. These observations suggest that cdiGRP plays a central role in the response to cadmium. Further microscopic examination of tissue sections of cadmium treated plants and transgenic plants overexpressing cdiGRP revealed that both plant groups had an increased callose accumulation in vascular bundles. Increased callose deposition has previously been associated with the constriction of plasmodesmata and decreased efficiency of viral movement (Iglesias & Meins 2000). The new findings concerning the cdiGRP-mediated deposition of callose suggest that accumulation of this material in the vasculature may be involved in regulation of systemic RNA silencing.

1.7 Factors affecting the efficiency of gene silencing

1.7.1 Suppressors of gene silencing

Several decades ago it was noticed that mixed viral infections that included a potyvirus were often accompanied by a dramatic increase in the severity of the symptoms (Rochow & Ross 1955, Goodman & Ross 1974a, Goodman & Ross 1974b, Pruss *et al.* 1997). This phenomenon was termed synergistic disease and was extensively studied using mixed PVX / potyviral infections. In such infections, the accumulation of PVX is increased by up to 10-fold without a corresponding change in the levels of the potyvirus (Goodman & Ross 1974a, Goodman & Ross 1974b, Vance 1991). The enhancement of PVX is due to an increase in replication in co-infected cells, rather than an increased number of PVX infected cells (Goodman & Ross

1974b). Subsequent work revealed that a conserved 5' potyviral sequence, encoding helper component protease (HC-Pro), was responsible for the increase in PVX replication *in planta* (Vance *et al.* 1995, Shi *et al.* 1997). Similar results were obtained in transgenic protoplasts expressing HC-Pro that were infected with PVX alone (Pruss *et al.* 1997).

It has subsequently been demonstrated that HC-Pro is an effective suppressor of PTGS in a variety of silencing systems (Anandalakshmi *et al.* 1998, Kasschau & Carrington 1998, Marathe *et al.* 2000a, Teycheney & Tepfer 2001). One of the most commonly used systems involves induction of PTGS in *Nicotiana benthamiana* plants expressing green fluorescent protein (GFP). Infection with recombinant PVX-GFP virus or agroinfiltration with a construct encoding GFP can induce systemic GFP silencing in such plants (Voinnet *et al.* 1998). However, infection with a recombinant PVX carrying both GFP and HC-Pro, exacerbates PVX symptoms and fails to initiate silencing. Furthermore, already established GFP silencing can be reversed by infection with a virus encoding HC-Pro (Anandalakshmi *et al.* 1998, Voinnet *et al.* 1999). Taken together, these observations suggest that HC-Pro can suppress both initiation and maintenance of PTGS.

The effect of HC-Pro on transcriptional gene silencing (TGS) was examined in an elegant series of experiments (Marathe *et al.* 2000b, Mette *et al.* 2001). Within the same plant, infection with an HC-Pro encoding potyvirus could effectively alleviate PTGS of nitrate reductase but had no effect on TGS of a *GUS* reporter gene (Marathe *et al.* 2000b). The same was observed when HC-Pro was expressed from a transgene. Interestingly, when HC-Pro itself was a target of TGS, initiated a short time after

germination, its transient expression could not maintain suppression of nitrate reductase PTGS (Marathe *et al.* 2000b). These results therefore indicate that HC-Pro affects neither the initiation nor the maintenance of TGS, and only its continuous expression can maintain suppression of PTGS.

Several studies attempted to determine the mode of action of HC-Pro (Johansen & Carrington 2001, Mallory *et al.* 2001, Mallory *et al.* 2003, Dunoyer *et al.* 2004). Mallory *et al.* (2001) reported that HC-Pro suppressed GUS silencing in transgenic plants and also eliminated the associated siRNAs. However, the same group found in a later study that HC-Pro suppressed accumulation of 21-24nt siRNA but did not affect accumulation of longer (25-27nt) siRNAs (Mallory *et al.* 2003). Johansen & Carrington (2001) investigated the effect of HC-Pro on PTGS in a system where PTGS of green fluorescent protein (GFP) is induced by agro-infiltration of single stranded GFP (ssGFP) or hpRNA GFP transgenes. These authors found that HC-Pro did not prevent the formation of siRNAs. Dunoyer *et al.* (2004) investigated the effect of HC-Pro on hpRNA-mediated silencing and reported that HC-Pro decreased the efficiency of dsRNA processing resulting in lower levels, but not complete elimination, of siRNAs. Taken together, these findings suggest that HC-Pro interferes with the activity of RISC (Silhavy & Burgyan 2004). If this were the case, some siRNAs would still be produced as a result of Dicer activity, but the production of secondary siRNA resulting from mRNA cleavage would be suppressed.

A number of other suppressors of gene silencing, encoded by a variety of viruses, have now been identified (Table 1.2) (Beclin *et al.* 1998, Voinnet *et al.* 1999). These proteins are likely to act on diverse points in the gene silencing pathway. For example,

Table 1.2: Known and putative suppressors of gene silencing and their effect on PTGS. (adapted from Voinnet *et al.*1999)

Virus group	Virus	Effect				Protein
		Reversion of established PTGS in old leaves	Inhibition of establishment of PTGS in new leaves	Suppression		
				whole leaf	veins only	
Comovirus	CpMV	✓	✓		✓	?
Cucumovirus	CMV		✓	✓		2b
Geminivirus	ACMV	✓	✓	✓		AC2
Potexvirus	PVX		✓	✓		p25
Potyvirus	PVY/TEV	✓	✓	✓		HC-Pro
Tobamovirus	TMV	✓	✓		✓	?
Tobravirus	TRV	✓	✓	✓		?
Tombusvirus	TBSV		✓		✓	19K

25p, a suppressor of PTGS encoded by PVX, is proposed to act by preventing the spread of the silencing signal out of infected cells (Voinnet *et al.* 2000). Similarly, 2b, a protein encoded by the cucumber mosaic virus, inhibits the initiation of silencing in plant meristem tissue (Ding *et al.* 1995, Brigneti *et al.* 1998). In contrast to HC-Pro, neither of these proteins can counteract already established PTGS (Brigneti *et al.* 1998, Voinnet *et al.* 1999, Voinnet *et al.* 2000). It may be that severe synergistic disease results from the interaction of viruses that suppress gene silencing at different points in the pathway and more completely overcome the plant's defence mechanism.

1.7.2 Environmental conditions

The effect of environmental conditions on gene silencing was first noticed by Dorlhac de Borne *et al.* (1994) while investigating transgene-induced co-suppression of nitrate reductase. The authors observed that early onset of co-suppression could be induced by high light intensities and abundant nutrient supply. In a follow-up study, Palauqui *et al.* (1996) found that the percentage of isogenic plants affected by co-suppression is higher when the plants are grown *in vitro* than when they are grown in the field. *In vitro* conditions are characterised by high light intensities (120 mE m^2 s^{-1}), abundant nutrient and a constant temperature of 24°C. Although neither of these studies specifically examined the effect of temperature on co-suppression, it is interesting to note that the plants grown in a field were likely to be exposed to an average temperature of 17°C.

The effect of temperature on gene silencing was more closely examined by two recent studies. Kalantidis *et al.* (2002) generated tobacco plants expressing an inverted repeat of a cucumber mosaic virus (CMV) derived sequence. Plants in which

expression of this transgene resulted in the production of homologous siRNAs were immune to CMV. However, when authors quantified siRNA production they observed that siRNAs accumulated to a much higher level in plants grown at 32°C than in those grown at 25°C.

Szittya *et al.* (2003) conducted a very thorough study examining the effect of temperature on virus-induced and transgene-induced PTGS. Production of virus-derived siRNAs was quantified during Cymbidium ringspot virus (CymRSV) infection of wild-type *Nicotiana benthamiana* protoplasts at 15°C, 21°C, 24°C and 27°C. siRNAs were abundant at 27°C, but undetectable at 15°C with the amount of siRNAs gradually increasing with temperature. Since virus-specific siRNAs are derived from dsRNA intermediates of viral replication (Voinnet 2001), and since CymRSV replicates efficiently at 15°C (Szittya *et al.* 2003), the lack of siRNAs at 15°C could be due to reduced Dicer activity at low temperatures.

Systemic infection of *Nicotiana benthamiana* by CymRSV is also affected by temperature (Szittya *et al.* 2003). At temperatures from 15°C, 21°C and 24°C CymRSV systemically infects these plants and kills them within two weeks. However, at 27°C virus levels are reduced and symptoms attenuated. At higher temperatures CymRSV with a mutation in p19, a suppressor of gene silencing, is unable to systemically infect *N. benthamiana* plants, but at 15°C it causes strong viral symptoms. These observations suggest that the PTGS-based response to viral infection is enhanced at higher temperatures and dramatically reduced at lower temperatures, and could provide an explanation for frequent outbreaks of plant virus diseases in unusually cold seasons.

Szittya *et al.* (2003) also examined the effect of temperature on transgene-induced silencing in diverse experimental systems. They found that agro-infiltration induced transient silencing, transgene-mediated virus resistance in *N. benthamiana* and silencing of endogenous genes in *A.thaliana* and potato were all inhibited at low temperatures. Inhibition of silencing was accompanied by an increase in the level of target mRNA and the loss of siRNAs (Szittya *et al.* 2003).

Taken together, these findings demonstrate the need for consideration of environmental factors when developing further biotechnological applications of RNA-based silencing.

1.7.3 Developmental factors

The effect of the developmental stage on the establishment of gene silencing was first documented by Kunz *et al.* (1996). These authors noticed that transgene-mediated silencing of chitinase in tobacco plants becomes established at the 6-10 leaf stage. Following its onset, gene silencing is stable in mature plants. Chitinase activity is restored in the progeny of plants exhibiting silencing until the plantlets reach the 6-10 leaf stage, when silencing is re-established.

Transgene-mediated virus resistance also appears to be less effective in very young plants. Lettuce plant lines expressing a transgene derived from tomato-spotted wilt virus (TSWV) are resistant to TSWV if inoculated at the 7-8 leaf stage. However, isogenic plants inoculated at the 4-5 leaf stage are susceptible to TSWV (Pang *et al.* 1996). It appears that the increased susceptibility of very young plants is due to the

reduced efficacy of gene silencing mechanisms during early developmental stages. This hypothesis is supported by the finding that tobacco plants expressing a CMV derived sequence in a hairpin conformation exhibit reduced accumulation of siRNAs up to the 7-leaf stage (Kalantidis *et al.* 2002).

The ability of the silencing signal to induce the spread of silencing into previously unaffected plant tissues also appears to be dependent on the age of the target tissue. For example, when co-suppression of nitrite reductase is triggered immediately post-germination, it rapidly spreads from a small area to the entire plant. However, when co-suppression is triggered as late as 50 days post-germination, its spread is much slower and incomplete, resulting in a variegated appearance (Boerjan *et al.* 1994, Elmayan & Vaucheret 1996, Palauqui *et al.* 1996). Furthermore, Crete *et al.* (2001) found that the efficiency of silencing mediated by biolistic introduction of a chitinase gene was strongly influenced by the age of the bombarded plants. Systemic chitinase silencing could only be induced in tobacco plants that had less then nine leaves.

Taken together these findings suggest that the establishment of gene silencing is highly dependent on the developmental stage of the plant. Transgene-mediated silencing appears to be less efficient in very young plants, while the spread of silencing into older plant tissues is slow and patchy.

1.8 Applications of gene silencing

Gene silencing was initially viewed as an obstacle to the new era of transgenics that promised better yields and more nutritious produce. However, it has now become

apparent that gene silencing is a powerful tool for both applied and basic plant science (reviewed by Wang & Waterhouse 2002).

Wang *et al.* (2000) have designed a hairpin RNA construct that confers robust and stable immunity to one of the most widespread cereal viruses - barley yellow dwarf virus. The same technology could be used to create silencing constructs targeting multiple viruses that may threaten valuable crop plants. Silencing is also an attractive method for the selective inhibition of endogenous genes, including genes encoding enzymes involved in multi-step metabolic pathways. For example, cotton-seed oil with an improved fatty acid profile has been produced by transgene-induced PTGS of specific destaturase genes (Singh *et al.* 2000).

Plant functional genomics is another area of research that has the potential to benefit from gene silencing technology. Although the genome of *A.thaliana* is now sequenced, biological function has been assigned to only a small proportion of genes. To date, studies of gene function in *Arabidopsis* were mostly based on loss of function phenotypes generated by insertion, chemical or radiation mutagenesis. These approaches have a number of limitations including their non-specific nature, difficulties in mapping mutations and the inability to study genes whose complete loss of function is lethal. However, most of these limitations can be overcome by using gene-silencing technology. The development of a vector system that allows the one-step generation of hairpin RNA constructs (Wesley *et al.* 2001), combined with efficient *A. thaliana* transformation, is likely to produce high throughput functional analyses similar to those currently underway for *C. elegans* (Fraser *et al.* 2000, Gonczy *et al.* 2000, Kamath & Ahringer 2003, Vastenhouw *et al.* 2003). Another

attractive feature of this approach is that constructs can be placed under the control of an inducible promoter (Guo *et al.* 2003) or can be designed to reduce, rather than completely inhibit, the expression of the target gene, thus allowing for the analysis of genes that are null-lethal (Levin *et al.* 2000). Conversely, constructs can be designed to target conserved regions of a gene family, thereby overcoming the problem of no change in phenotype in plants carrying a mutation in one member of a gene family. Some of these strategies have already been successfully employed to study genes involved in flower development and methionine biosynthesis (Levin *et al.* 2000, Chuang & Meyerowitz 2000).

Virus-induced gene silencing (VIGS) is another fast and effective way of establishing gene function (reviewed by Baulcombe 1999). VIGS operates by the infection of a plant with a recombinant virus carrying sequences homologous to a target gene. This eliminates the need for plant transformation and speeds up the process of phenotype identification (Burton *et al.* 2000). The RNA genomes of these viruses can be inoculated directly onto a plant or can be cloned into binary vectors and delivered for transient expression mediated by agro-infiltration. VIGS was pioneered by the development of tobacco mosaic virus (TMV) and potato virus X (PVX) vectors (Kumagi *et al.*1995, Ruiz *et al.* 1998). Although effective, these recombinant viruses have a number of disadvantages. Firstly, the symptoms of TMV and PVX are quite severe and may obscure the silenced phenotype (Ratcliff *et al.* 2001). Secondly, these viruses do not produce uniform infections, resulting in a mosaic-silencing pattern. Finally, TMV and PVX are excluded from meristem tissue, which renders them ineffective for silencing of genes that may be active in rapidly growing tissues. Some of these disadvantages have been addressed by the recent development of tobacco

rattle virus (TRV) and tomato golden mosaic virus (TGMV) vectors (Peele *et al.* 2001, Ratcliff *et al.* 2001). Both vectors are able to induce silencing in meristematic tissue. TRV also produces uniform infections with very mild symptoms (Ratcliff *et al.* 2001). With these improvements, VIGS has certainly become an attractive approach for "fast-forward" functional genomics.

1.9 Scope of this thesis

The insights into gene silencing gained over the last decade have not only given us better understanding of plant defence and gene regulation mechanisms, but they have also given us powerful new tools. Yet, our understanding of gene silencing is far from complete. This thesis aims to further explore the mechanism of gene silencing.

Chapter two: Our laboratory has previously demonstrated that transgene-mediated virus resistance against potato virus Y (PVY) can be achieved in transgenic *Nicotiana tabacum* plants by co-expression of complementary PVY-derived sense and antisense RNAs, or by expression of the same PVY sequence arranged as a self-complementary, hairpin RNA (hpRNA) (Waterhouse *et al.* 1998, Smith *et al.* 2000). In addition, cross-protection against severe PVY strains can also be obtained by prior infection with a mild PVY strain. This chapter compares the characteristics of these three approaches to virus resistance and provides insights into the mechanism of RNA silencing in plants.

Chapter three: Grafting experiments have previously been used to study the spread of silencing from plant tissues affected by PTGS into the unaffected (active) plant tissues. The results varied depending on the grafting approach and the silencing

system. This chapter aims to examine the effect of plant age, transgene structure and inducer/target sequence homology on the systemic spread of PTGS and to continue the comparison between hpRNA and S+AS transgenes.

Chapter four: One of the striking features of gene silencing is that perfect homology between dsRNA and its single stranded target is not necessary for induction of highly effective silencing. This observation suggests that siRNAs may not only be used as guides for an immediate cleavage of target RNA; but they could also act as primers for its amplification. Amplification could generate additional dsRNA and, upon its cleavage, many new siRNAs that are completely homologous to the target RNA (Lipardi *et al.* 2001, Sijen *et al.* 2001a). In addition, the amplification could also result in extension of silencing into sequences upstream and/or downstream of the target sequence.

siRNAs also guide methylation of homologous DNA sequences. As such, DNA methylation can be used as an indicator of the presence of homologous siRNA species. This chapter describes the mapping of methylation patterns associated with PTGS and TGS and use of these methylation maps to determine whether the amplification and extension of silencing into neighbouring sequences occurs in these two forms of silencing.

Chapter five: This concluding chapter summarises the experimental findings described in chapters two, three and four and explores possibilities for further research.

Chapter 2

Characterisation of cross-protection and transgene-mediated virus protection

2.1 Introduction

2.1.1 Aims

The experiments described in this chapter compare three methods of plant protection against potato virus Y (PVY) infection. The first is based on cross protection, the second relies on simultaneous expression of PYV-derived transgenes in sense and antisense (S+AS) orientations while the third is mediated by the same PVY derived sequence, but this time expressed in a hairpin conformation (hpRNA). This chapter examines the breadth of protection each of these methods offers, investigates the effect of a suppressor of gene silencing on the efficiency of the protection and analyses the effect of plant age on the dynamics of transgene-mediated protection.

2.1.2 Virus protection

For many decades plant viruses have been a major problem in the cultivation of crops throughout the world. In the past these pathogens have been controlled using conventional measures like crop rotation, destruction of infected source plants, breeding for resistance and chemical control of insect vectors. A better understanding of the molecular genetics of plant viruses and plant defence systems has resulted in the development of novel ways to control virus diseases in plants (reviewed in Goldbach *et al.* 2003).

2.1.2.1 Cross protection

Cross protection is a phenomenon in which systemic infection of a plant by one virus prevents infection with another, closely related, virus. This phenomenon was first reported by McKinney (1929) who noticed that plants inoculated with a mild strain of tobacco mosaic virus (TMV) did not develop additional symptoms when challenged

with a yellow mosaic strain. Since its discovery cross protection has been used in plant virus classification and as a disease control measure (Cassells & Herrick 1977).

A number of hypotheses describing the mechanisms of cross protection have been put forward, including those proposing that cross protection is coat protein (CP) or RNA-mediated. Involvement of the CP is supported by the data showing that expression of TMV CP containing mutations that enhance inter-subunit interactions and favor helical aggregation results in better cross protection than that offered by wild type CP (Lu *et al.* 1998). However, the observation that a mild strain of potato spindle tuber viroid (PSTV), which does not encode proteins, can cross-protect against a severe PSTV strain suggested that cross protection could be RNA-mediated (Niblett *et al.* 1978).

More recent studies have generated evidence indicating that cross-protection may operate via a mechanism related to PTGS. Ratcliff *et al.* (1999) demonstrated that a recombinant tobra-virus carrying a green fluorescent protein (GFP) derived sequence can cross protect against a potex-virus carrying a portion of GFP sequence. In addition, these viruses could induce silencing of transiently expressed GFP (Ratcliff *et al.* 1999). Conversely, transgenic plants with pre-established PTGS have been shown to be resistant to recombinant viruses encoding sequences homologous to the silenced gene. For example, PVX encoding a segment of the *GUS* gene is targeted by PTGS in plants carrying a post-transcriptionally silenced *GUS* transgene and is therefore unable to replicate in such plants (English *et al.* 1996).

A hypothesis consistent with these observations is that the first virus replicates and spreads through the plant leaving behind siRNAs derived from its genome and activated PTGS machinery. When the plant is challenged with the second, closely related virus, the already primed PTGS machinery eliminates it.

2.1.2.2 Transgene mediated protection

Early attempts to engineer virus resistance using transgenes involved the expression of viral coat proteins in target plants (reviewed in Wilson 1993, Hackland *et al.* 1994, Miller & Hemenway 1998, Beachy 1999). This approach was extended to the expression of viral replicase and movement proteins (Carr & Zaitlin 1991, Audy *et al.* 1994, Carr *et al.* 1994, Cooper *et al.* 1995). This type of transgene-mediated resistance often relied on the expression of defective viral proteins which were believed to interfere with replication or movement of the invading virus. These transgenes usually offered broad protection against viruses related to the strain from which the transgene was derived (reviewed in Beachy 1996). However, in most cases, the protection was incomplete allowing for some level of virus accumulation.

Current strategies for generating virus resistant plants rely on the expression of non-translatable virus-derived RNA sequences. Expression of either sense (S) or antisense (AS) sequences does not yield consistent results. However co-expression of S and AS RNAs can produce robust virus resistance (Waterhouse *et al.* 1998). The efficiency of virus resistance appears to be further improved by the expression of virus-derived sequences in the form of hairpin RNA (hpRNA) (Smith *et al.* 2000, Wesley *et al.* 2001). Both S+AS and hpRNA-mediated virus resistance are thought to operate via a PTGS-based mechanism. In comparison to protein-based resistance, PTGS-based

resistance appears to be more specific to the viral strain from which the transgene was derived (Maki-Valkama *et al.* 2000).

2.1.3 Experimental system

2.1.3.1 Viruses

Potato virus Y (PVY) is a member of the genus *Potyvirus* in the family *Potyviridae*. Its monopartite genome is composed of single stranded RNA molecule of ~9.7kb. During the infection process, viral RNA is translated into a large precursor polyprotein which is cleaved into 10 mature proteins (Dougherty 1988, Riechmann 1992).

PVY is a common pathogen of potato worldwide and can reduce yields by up to 80%. There are three main groups of PVY strains: PVY^O (ordinary type), PVY^C and PVY^N (necrotic type). In potato plants symptoms of PVY^O and PVY^C include leaf mottling, yellowing, defoliation and premature stem death while PVY^N produces milder symptoms of leaf mottling. PVY strains also infect capsicum, tomato and tobacco crops. *N. tabaccum* is a well-known experimental host species for PVY. PVY^O symptoms in *N. tabaccum* include mild mottling symptoms while PVY^N infection results in severe leaf and stem necrosis (Jongedijk *et al.* 1993, Jones *et al.* 2003).

2.1.3.2 PVY-resistant plant lines

N. tabaccum plant lines expressing an untranslatable NIa protease gene from potato virus Y, strain D (PVY-D) in sense (S) and/or antisense (AS) orientation were previously generated in our laboratory (Waterhouse *et al.* 1998). Plants expressing this virus-derived transgene in both the sense and antisense orientations, termed

S+AS plants, were found to be immune to the PVY-D strain from which the transgene was derived (Waterhouse *et al.* 1998).

More recently, genome-integrated transgenes encoding hairpin RNA (hpRNA) have been used to induce silencing of endogenous and viral genes (Smith *et al.* 2000, Wesley *et al.* 2001). hpRNA constructs derived from the PVY-D NIa gene proved to be very efficient at mediating resistance to this virus.

In order to confirm that resistance of S+AS and hpRNA plants to PVY-D results from transgene-induced PTGS, RNA species present in these plant lines were analysed (Neil Smith *pers. comm.)*. Total RNA was extracted from hpRNA plants, S+AS plants, parental S plants, parental AS plants and non-transgenic plants, and subjected to northern blot analysis. None of these plants had been inoculated with PVY. Transgene mRNAs of the expected sizes and polarity were detected (Fig. 2.1 A, B). High levels of PVY-specific siRNA (Fig. 2.1 C) were detectable in the hpRNA plants, much lower levels were found in the S+A/S plants and there were no detectable siRNAs in the control, S or A/S plants. The accumulation of siRNA species homologous to the hpRNA and the S+AS transgenes indicates that these transgenes induce PTGS which in turn results in the development of resistance to the homologous virus. In addition, these findings suggest that the hpRNA transgene is a more efficient trigger of PTGS than the separately expressed S+AS transgenes.

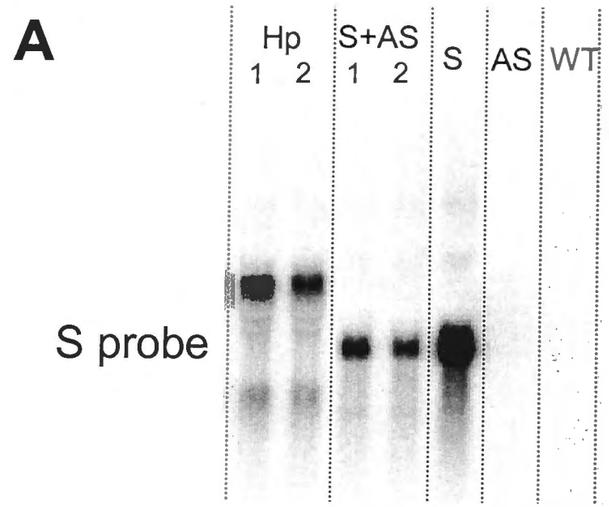
Figure 2.1: Analysis of RNA species in wild S+AS, hpRNA and wild type plants.

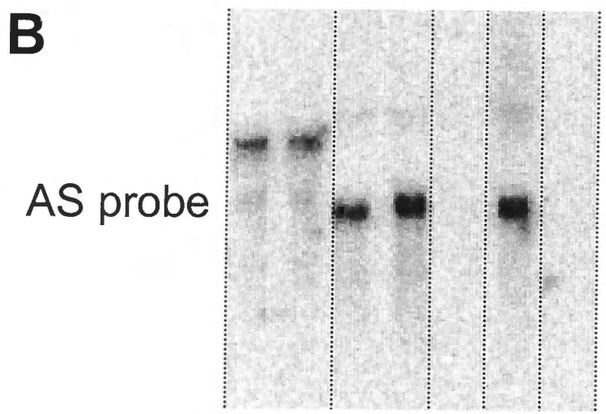
A – B Total RNA was extracted from S+AS, hpRNA, S, AS and wild type (WT) plants, run on an agarose gel and transferred onto a nylon membrane. The membrane was probed with radioactively labelled probes derived from the NIa-Pro constructs cloned in sense (A) and antisense (B) orientations.

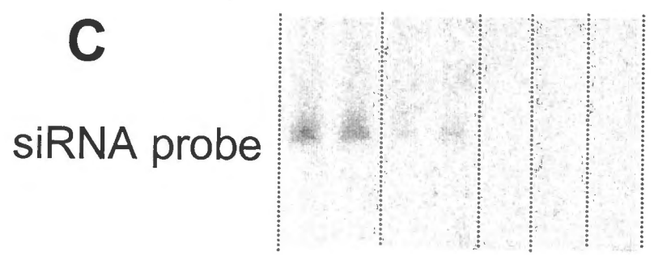
C Small RNAs were extracted from S+AS, hpRNA and wild type plants, electrophoresed on an acrylamide gel and transferred to a nylon membrane. The membrane was probed with a radioactively labelled siRNA probe derived from the NIa-Pro constructs cloned in sense orientation.

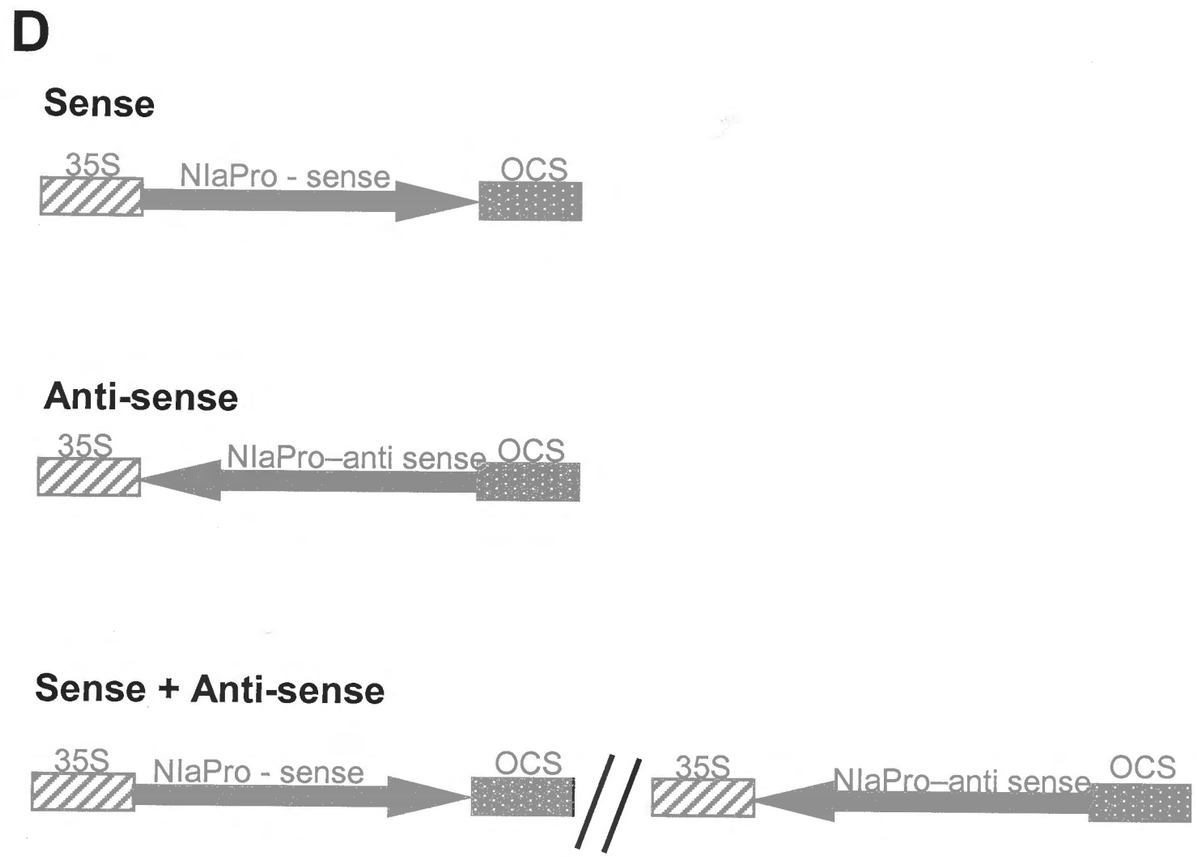
D Schematic representation of S, AS, S+AS and hpRNA constructs.

Abbreviations:
35S: promoter derived from the Cauliflower Mosaic Virus
OCS: octopine synthase terminator sequence
PDK: pyruvate orthophosphate dikinase
NIa-Pro: NIa protease gene from Potato Virus Y


A
Hp
1 2
S+AS
1 2
S
AS
WT
S probe


B
AS probe


C
siRNA probe


D
Sense
35S
NIaPro - sense
OCS
Anti-sense
35S
NIaPro–anti sense
OCS
Sense + Anti-sense
35S
NIaPro - sense
OCS
35S
NIaPro–anti sense
OCS
Hairpin
35S
NIaPro - sense
PDK intron
NIaPro–anti sense
OCS

2.1.3.3 HC-Pro expressing plant line

Potyviruses encode a single polypeptide that self-processes to release all the viral proteins needed to establish a systemic infection. One of these is the helper component proteinase (HC-Pro).

HC-Pro performs multiple functions during the infection cycle. It is essential for viral transmission by insect vectors, acts as an enhancer of infectivity and genome amplification and plays an important role in cell-to-cell and systemic viral movement (Oh & Carrington 1989, Kasschau *et al.* 1997). In addition, HC-Pro has been identified as a suppressor of PTGS (discussed in 1.7.1). This finding raised the possibility that expression of HC-Pro from a viral genome may compromise transgene-mediated virus resistance. However, in transgenic virus-resistant plants, viral RNA appears to be degraded before the viral-encoded HC-Pro protein can be synthesised to a level capable of suppressing the protection.

In plants expressing HC-Pro from a transgene integrated into the genome, the HC-Pro protein would be present prior to viral infection. The effect of *in planta* expressed HC-Pro on PTGS of GUS transgenes has previously been investigated. The authors found that HC-Pro altered the processing of double stranded GUS RNA and relieved GUS silencing (Mallory *et al.* 2001, Mallory *et al.* 2002).

Little is currently known about the effect of *in planta* expressed HC-Pro on cross protection and transgene-mediated virus protection. In this study the effect of HC-Pro on virus protection was investigated using *Nicotiana tabaccum* U-6B line

carrying HC-Pro sequence from tobacco etch virus (TEV) (Carrington *et al.* 1990, Mallory *et al.* 2001).

2.2 Materials and methods

2.2.1 Plants and viruses

The S+AS and HpRNA tobacco plants lines used in this study have been previously described (Waterhouse *et al.* 1998, Smith *et al.* 2000). The NS12 S+AS line contains 280bp from the 5' end of the transgene used to make the original S+AS lines (Fig. 2.8). Tobacco plant line U6-B expressing wild type HC-Pro and plant line transformed with the vector backbone only were provided by Dr Vicky Vance (North Carolina State University, USA). These plant lines were previously described by Mallory *et al.* (2001).

A tobacco plant line expressing β-galactosidase (*GUS*) was generated and characterised by Lisa Molvig (CSIRO Plant Industry, Australia). The tobacco plant line 8.7, expressing hairpin RNA transgene derived from the *GUS*, gene was made and characterised by Dominikus Akhadi (CSIRO Plant Industry, Australia).

PVY strains (D, 18S1, 431S, 120F and 55N) were provided by Dr John Thomas (QDPI, Indooroopilly, Australia). An alfalfa mosaic virus (AMV) strain was provided by Dr Paul Chu, CSIRO Plant Industry, Australia. PVY strains were propagated in tobacco while AMV was propagated in white clover plants. Glasshouse temperatures were 25°C during the day and 20°C at night.

2.2.2 Isolation of viral RNA and sequencing of the NIa-Pro gene

Plants were inoculated with the virus of interest and two weeks post-inoculation, 2g of infected leaf tissue was harvested. The central veins were removed from the leaves and the remaining tissue homogenised with two volumes (w/v) of 0.2M phosphate buffer (pH 8.0) / 0.2% 2-mercaptoethanol / 0.01M ethylenediamine tetra-acetic acid disodium salt (EDTA). The extract was filtered through three layers of gauze and clarified by centrifugation at 7800g for 20 minutes. 1% of Triton X-100 was added to the supernatant and the mixture was stirred at 4°C for two hours. The clarification process was repeated and the supernatant was brought to a final concentration of 4% PEG (PM 8000) and 0.2M NaCl. The mixture was stirred at 4°C for 60 minutes and then incubated at room temperature for another 60 minutes. Viral particles were precipitated by a 20-minute centrifugation at 7800g. The resulting pellet was resuspended in 0.02M phosphate buffer (pH 7.2) / 1% Triton X-100 at 4°C overnight.

RNA was released from viral particles by combining equal volumes of purified virus and dissociation buffer (0.02M tris-HCl pH 9, 0.001M EDTA, 4% sodium dodecyl sulphate (SDS)). The mixture was heated at 60°C for 20 minutes and then emulsified with an equal volume of phenol. The phases were separated by centrifugation (3000g, 10 minutes), the aqueous phase removed, mixed with 2.5 volumes of absolute ethanol and kept at -20°C overnight. The RNA was precipitated by centrifugation (3000g, 10 minutes) and residual phenol removed by several 70% ethanol washes. The RNA was precipitated once more using 70% ethanol at -20°C overnight, resuspended in nuclease-free water and quantified by spectrophotometer.

100ng of viral RNA was used in a reverse transcriptase polymerase chain reaction (RT-PCR) using forward (GAA CTA AGG CAA ACT GGG CCA GC) and reverse (CTC TTC ATT GTC GCC ACA GCT TTG C) primers. The reactions were prepared according to the manufacturer's instructions using the QIAGEN© OneStep RT-PCR kit (Qiagen GmbH, Germany). The PCR products were subcloned into pGEM-T vector (Promega, USA) and their sequences determined using dye terminator sequencing and M13 sequencing primers (Applied Biosystems, USA; 373A DNA Sequencing System).

2.2.3 Subcloning of PCR products

2.2.3.1 Ligation

10µl of the ligation mixture contained ~50ng of pGEM-T Easy vector (Promega, USA), 10-100ng of the appropriate PCR product, 3U of T4 Ligase and 1× rapid ligation buffer provided with the pGEM-T Easy vector system. The reaction was prepared in 1.5ml microfuge tubes and incubated overnight at 16°C.

2.2.3.2 Transformation

1µl of the ligation mixture was added to 50 µl of electro-competent DH5α cells. The cells were placed in electroporation cuvettes (BioRad, USA) and electroporated at 2 kV. The cells were washed out of the cuvettes with 1ml of Leurea-Bertani (LB) medium and incubated at 37°C for 30 minutes to allow expression of the resistance gene. 200µl of the transformed cells was then plated out onto an appropriate selection plate.

2.2.3.3 Small-scale preparation of plasmid DNA

Single bacterial colonies were cultured in 3mL of LB medium containing the appropriate antibiotic, for 20 hours at 37°C with vigorous shaking. 1.5ml of bacterial culture was centrifuged (10000rpm, 5 minutes) and the supernatant was removed. From this point onwards the pellets were processed using the QIAGEN Plasmid mini-prep kit (Qiagen GmbH, Germany) according to the manufacturer's instructions.

2.2.3.4 Screening for recombinant plasmids

2µl of each plasmid mini-preparation was digested in a total volume of 20 µl (1× restriction buffer) with 5 U of *Eco*RI restriction enzyme at 37°C for 1.5 hours. The reaction was stopped by adding 10 µl of Ficoll stop mix (6% (w/v) Ficoll-400, 75mM EDTA (pH 8.0), 0.009% (w/v) bromophenol blue). 20µl of the digest was then electrophoresed on a 1.4% agarose gel. Positive clones were identified by the presence of an 800bp band.

2.2.4 DNA sequencing

400ng of purified plasmid DNA, 4pmol M13 reverse sequencing primer (AGC GGA TAA CAA TTT CAC ACA GGA) and 5µl of Big Dye™ Terminator mix (Applied Biosystems, USA) were combined and the total volume made up to 20µl with reverse osmosis purified (RO) water. Thermal cycling was performed as follows: 1 cycle of 95 °C / 2min and 25 cycles of 96°C / 20sec, 50°C / 15sec, 60°C / 4min.

The reaction was then mixed with 20µl 3M sodium acetate (pH 5.0) and 50µl absolute ethanol. The contents were vortexed, incubated at room temperature (RT) for 15 minutes and centrifuged in a bench centrifuge (13000rpm, 15 minutes). The

supernatant was promptly removed and the pellet washed with 250µl cold 70% ethanol. After another 10-minute spin in a bench centrifuge, the supernatant was removed and the pellet was air-dried. Sequencing products were sent for analysis either to the CSIRO Plant Industry sequencing facility or to the Australian Genome Research Facility (AGRF), University of Queensland.

2.2.5 Viral inoculations

1g of infected plant tissue was ground, using a mortar and pestle, in 20 ml cold 0.1M phosphate buffer (pH 7.2). Two young opposing leaves of the experimental plant were sprinkled with superfine (600 grit) carborundum powder (Avocado© research chemicals, UK) and inoculated by rubbing in 300 µl of the extract.

In cases where two viruses were co-inoculated, plant tissue infected by each of the viruses was ground separately. The extracts were then combined, bringing the total volume to 40 ml, and 300µl per leaf was inoculated as described above.

For each experiment, three wild type plants were grown in parallel with experimental plants. These plants were not inoculated and were used as negative controls.

2.2.6 Enzyme-linked immunosorbent assay (ELISA)

Leaf discs were sampled from the two leaves above the originally inoculated leaves and ground in two volumes (v/w) of extraction buffer (10.8 mM Na_2SO_3, 2% (w/v) PVP (MW 24-40000), 0.3mM NaN_3, 0.2% (w/v) powdered egg albumin, 2% Tween-20 in phosphate buffered saline (PBS)). The extract was further diluted (1:5) with the extraction buffer. 100µl of diluent was dispensed into 96-well micro-titre plates

coated with PVY antibody (2% (v/v) antibody (Agdia©, USA; cat no. SRA 2001/5000) in 0.15 M sodium carbonate, 0.35 M sodium bicarbonate, 0.3 mM NaN_3). The plates were incubated overnight at 4°C, the extract was then removed and the plates were washed with PBS/Tween buffer (0.05% Tween-20, 0.3mM sodium azide in PBS). Antibody conjugated to alkaline phosphatase (Agdia©, USA; cat no. SRA 2001/5000) was applied to each well and allowed to bind for three hours at 37°C. The plates were then washed and 100µl substrate solution (0.6g/ml P-Nitrophenyl Phosphate di-sodium, 0.2% bovine serum albumin, 2% PVP (MW 24-4000) in PBS) was applied. Plates were covered with aluminium foil and incubated for 20 minutes at room temperature. A Labsystems Multiscan© Plus scanner was used to read plates at 405nm.

ELISA for alfalfa mosaic virus (AMV) was performed using the same set of buffers and solutions. AMV antibodies were kindly donated by Dr Paul Chu, CSIRO Plant Industry, Australia. Both coating and enzyme-conjugated antibodies were used at 0.5µg/ml concentration. Sampling and grinding of samples was done as described above. Plant extracts were diluted 1:1000 with the extraction buffer.

Samples were considered positive for virus infection if the 405nm reading was greater than 2× the average reading for uninfected tissue samples (McLaughlin *et al.* 1981, Burrows *et al.* 1984). A red line on graphs presenting ELISA data marks 405nm absorbance above which tissue samples are considered to be infected with a virus.

2.2.7 GUS staining

Leaf discs were completely immersed in X-gluc stain [0.125% (w/v) 5-Bromo-4-Chloro-3-indoyl-β-D glucuronic acid, di-methyl sulfoxide 5% (v/v), 5mM ferri-cyanide, 5mM ferro-cyanide, 100mM sodium phosphate buffer (pH 7), 0.3% Triton - X 9v/v)] and incubated overnight at 37°C. The stain was then removed and replaced with 70% ethanol. Ethanol was changed several times until all chlorophyll was removed from the leaf discs.

2.2.8 Plant crosses

The anthers of the plant donating pollen were allowed to mature, while the anthers of the pollen recipient were removed as soon as the bud formed. Following the removal of anthers the bud was closed and allowed to mature. When the stigma reached maturity the bud was re-opened and the stigma was dusted with pollen collected from the pollen donor. The bud was then closed, labeled with the details of the cross and allowed to set seed.

2.3 Results

2.3.1 Comparison of cross protection and transgene mediated virus resistance

It has been suggested that cross-protection of one strain of virus by another is based on the induction of PTGS by the initial virus (Ratcliff *et al.* 1999). This proposal appears to contradict findings by Maki-Valkama *et al.* (2000) which indicate that transgene mediated resistance, which is also based on PTGS, is limited to the strain from which the transgene was derived. To investigate whether cross protection and

transgene-mediated resistance offer a similar breadth of protection, wild type, S+AS and hpRNA expressing plants were challenged with a suite of PVY strains.

Four different chlorotic strains of PVY^O (D, 18S1, 431S and 120F) were inoculated onto different sets of eight wild-type tobacco plants (Wisconsin 38). Two weeks after inoculation, all of the plants had chlorotic symptoms (Fig. 2.2 A, B) and the infections were confirmed by enzyme-linked immunosorbent assay (ELISA). These infected plants, and an additional eight healthy plants, were inoculated with a necrotic PVY strain, 55N. Four weeks later, the plants were examined for symptoms. Plants challenged first with 18S1, 431S or 120F strains and then with 55N strain showed chlorotic but no necrotic symptoms (Fig. 2.2 E, F, G). Some necrotic spots were detected on plants first infected with the D strain and then challenged with 55N (Fig. 2.2 D). However, these symptoms were very mild compared to the severe necrosis observed in plants solely inoculated with the 55N strain (Fig. 2.2 H). After a further 4 weeks, the plants originally challenged with 18S1, 431S or 120F, and then with 55N, had no necrotic symptoms, while those inoculated with D and then with 55N retained some necrotic flecks. Plants solely challenged with 55N had developed severe necrosis of the stem and veins, and subsequently died. In conclusion, all of the tested PVY^O strains cross-protect against infection by the necrotic strain, 55N.

Tobacco plant lines carrying transgenes encoding sense and antisense (S+AS) RNAs or a hpRNA, derived from the PVY-D NIa protease gene, have previously been reported to be highly resistant to infection by PVY-D (Waterhouse *et al.* 1998, Smith *et al.* 2000). To test whether these transgenes could provide protection against other PVY strains, five sets of medium-sized (6 weeks old, with ~10 true leaves) plants

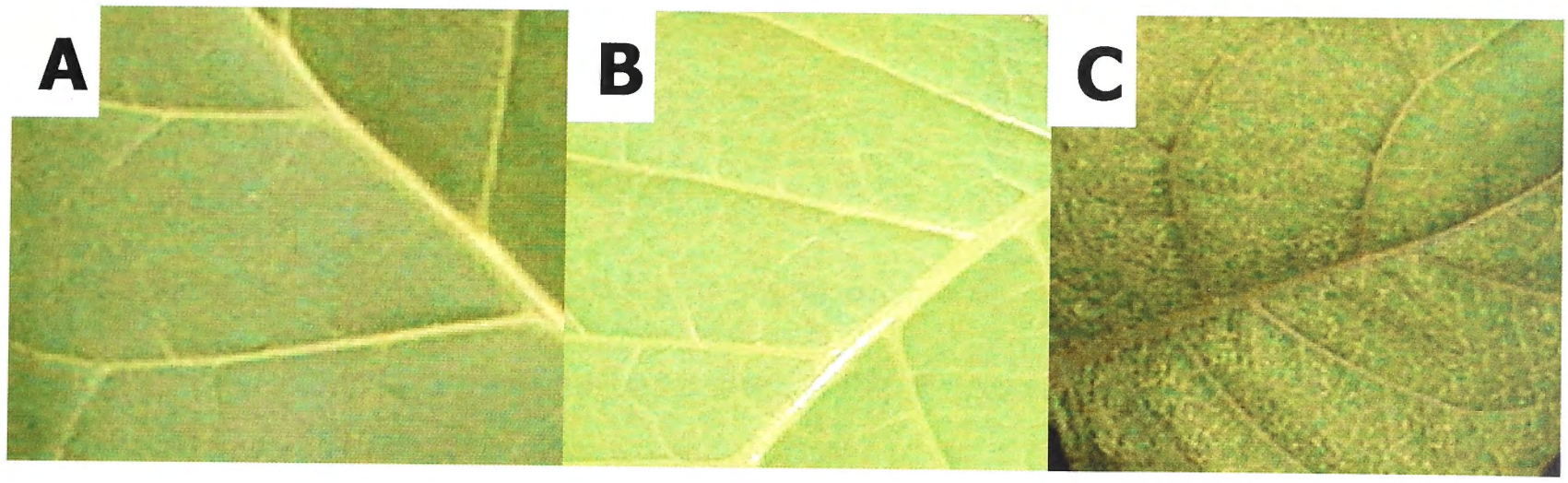


Figure 2.2: Cross protection mediated by PVY ordinary strains against a PVY necrotic strain.

A – C Appearance of healthy (A), PVY^{O}-infected (B) and PVY^{N}– infected (C) tobacco plants.

D - G Representative leaves harvested from plants that were inoculated with one of the ordinary PVY strains (D, 18S1, 431S or 120F), and afterwards challenged with the 55N necrotic strain.

H Representative leaf from a plant infected with the 55N necrotic strain.

carrying either S+AS or hpRNA transgene and five sets of non-transgenic control plants, were each challenged with one of the five PVY strains used in the cross protection experiments (D, 18S1, 431S, 120F and 55N). Three weeks after inoculation, all five PVY strains had accumulated to high levels, as assayed by ELISA, and caused disease symptoms in the non-transgenic plants. However, none of the PVY strains caused symptoms or accumulated to a detectable level in either the S+AS or hpRNA plants (Fig. 2.3). These results suggest that, like cross protection, S+AS and hpRNA constructs are able to mediate virus resistance against divergent PVY strains.

S+AS and hpRNA mediated protection is known to operate via a PTGS mechanism (section 2.1.3.2). As such, HC-Pro, a known suppressor of gene silencing, would be expected to interfere with S+AS and hpRNA mediated protection. Furthermore, if transgene mediated protection and cross-protection operate via a related mechanism then both would be similarly affected by HC-Pro. To further investigate this proposal plants containing a transgene encoding HC-Pro were crossed with S+AS and hpRNA plants. A cross between plants carrying an empty vector backbone (Vec), but not the HC-Pro gene, and S+AS and hpRNA plants was used as a control. Sets of eight progeny plants, with each set containing plants with one of the desired transgene combinations [HC-Pro + (S+AS), HC-Pro + hpRNA, Vec + (S+AS) or Vec + hpRNA], were challenged with PVY-D and assessed for infection three weeks later (Fig. 2.4). Plants carrying Vec + (S+AS) or Vec + hpRNA were immune to PVY-D, however, all plants that inherited S+AS or hpRNA transgene together with the HC-Pro transgene were susceptible to PVY-D (Fig. 2.4, Fig. 2.5 D, E, F, G). The susceptible plants were allowed to self-pollinate and the progeny were tested for

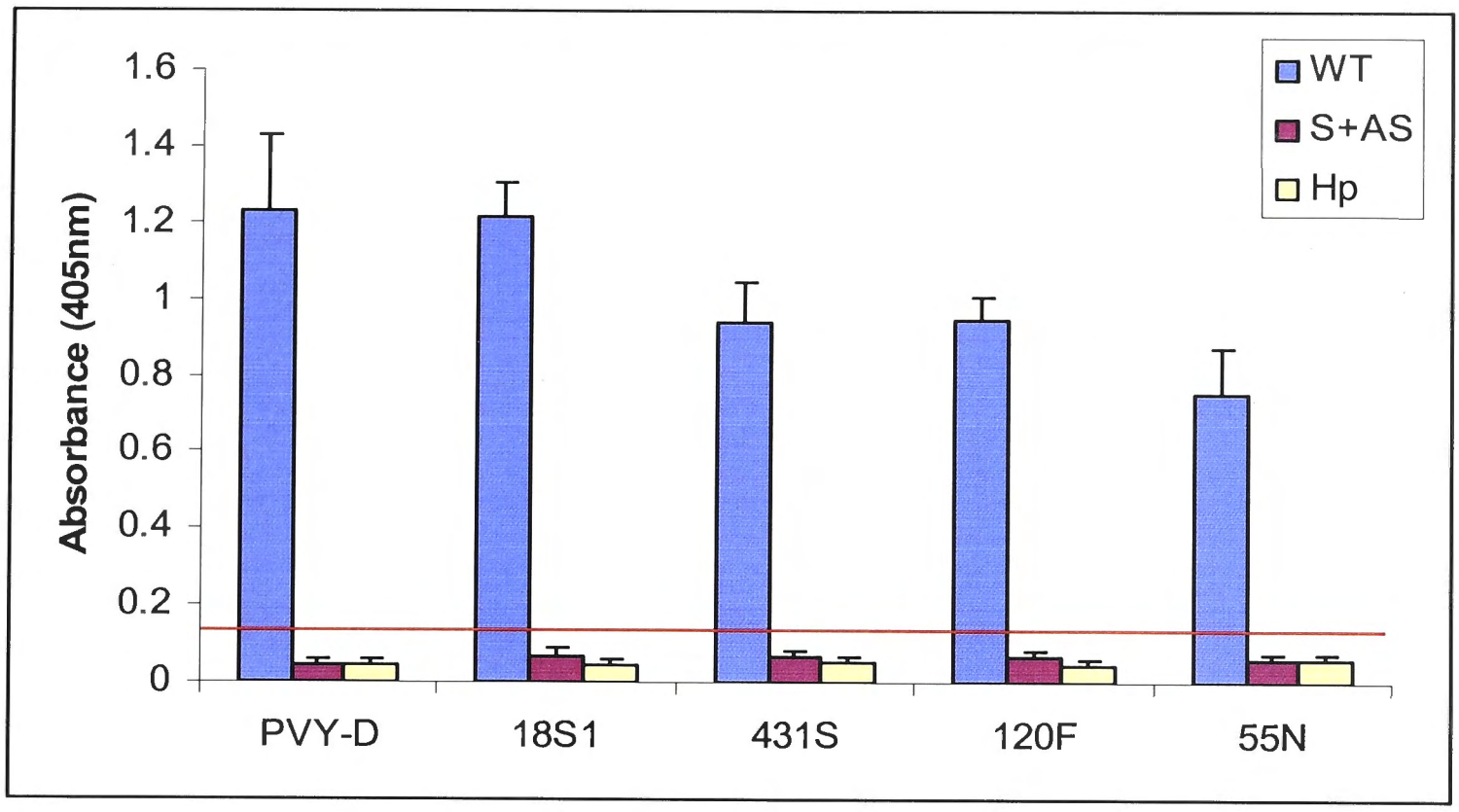


Figure 2.3: Comparison of viral levels in medium-sized wild type (WT), S+AS and hpRNA plants inoculated with PVY isolates.
Eight WT, S+AS and hpRNA plants were manually inoculated with one of the PVY strains. Virus levels were measured by ELISA at three weeks post-inoculation. Absorbance at 405nm positively correlates with viral titer. The columns represent median values while error bars are derived from standard error values. The horizontal line marks 405nm absorbance that equals to 2× the average absorbance value for uninfected tissue samples. Tissue samples are considered to be infected with a virus if their 405nm absorbance value is above the level marked with the horizontal line.

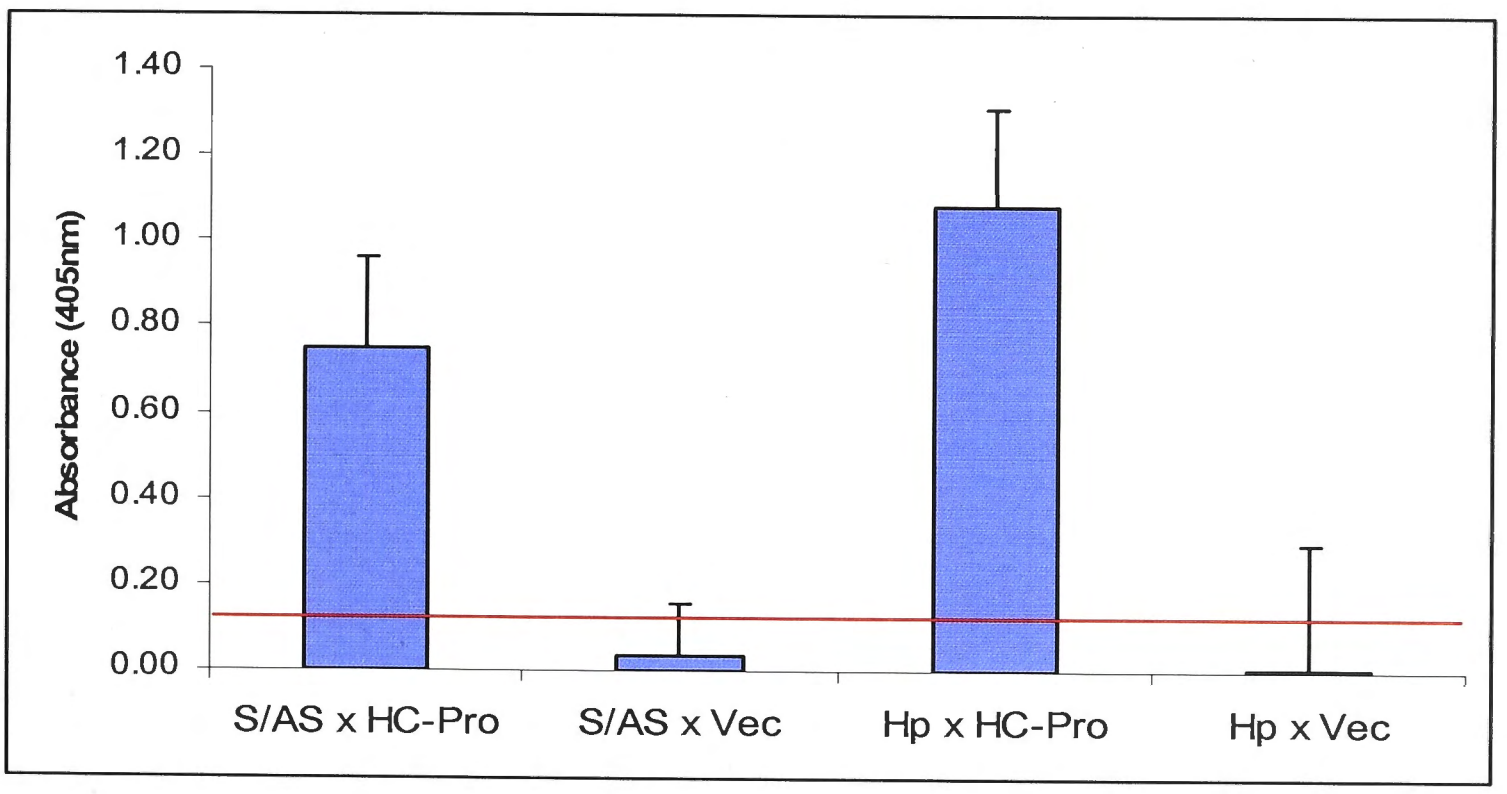


Figure 2.4: Viral levels in S+AS and hairpin plants carrying either HC-Pro transgene or an empty vector (Vec).
Eight S+AS x HC-Pro, S/AS x Vec, Hp x HC-Pro and Hp x Vec plants were inoculated with PVY-D strain. Virus levels were measured by ELISA at three weeks post-inoculation. The columns represent median values while error bars are derived from standard error values. The horizontal line marks 405nm absorbance that equals to 2× the average absorbance value for uninfected tissue samples. Tissue samples are considered to be infected with a virus if their 405nm absorbance value is above the level marked with the horizontal line.

Figure 2.5: Effect of HC-Pro on cross protection and transgene-mediated virus resistance.

Representative leaves from:

A Wild type plant inoculated with PVY-D and, two weeks later, with the 55N strain

B HC-Pro expressing plants inoculated with 55N only

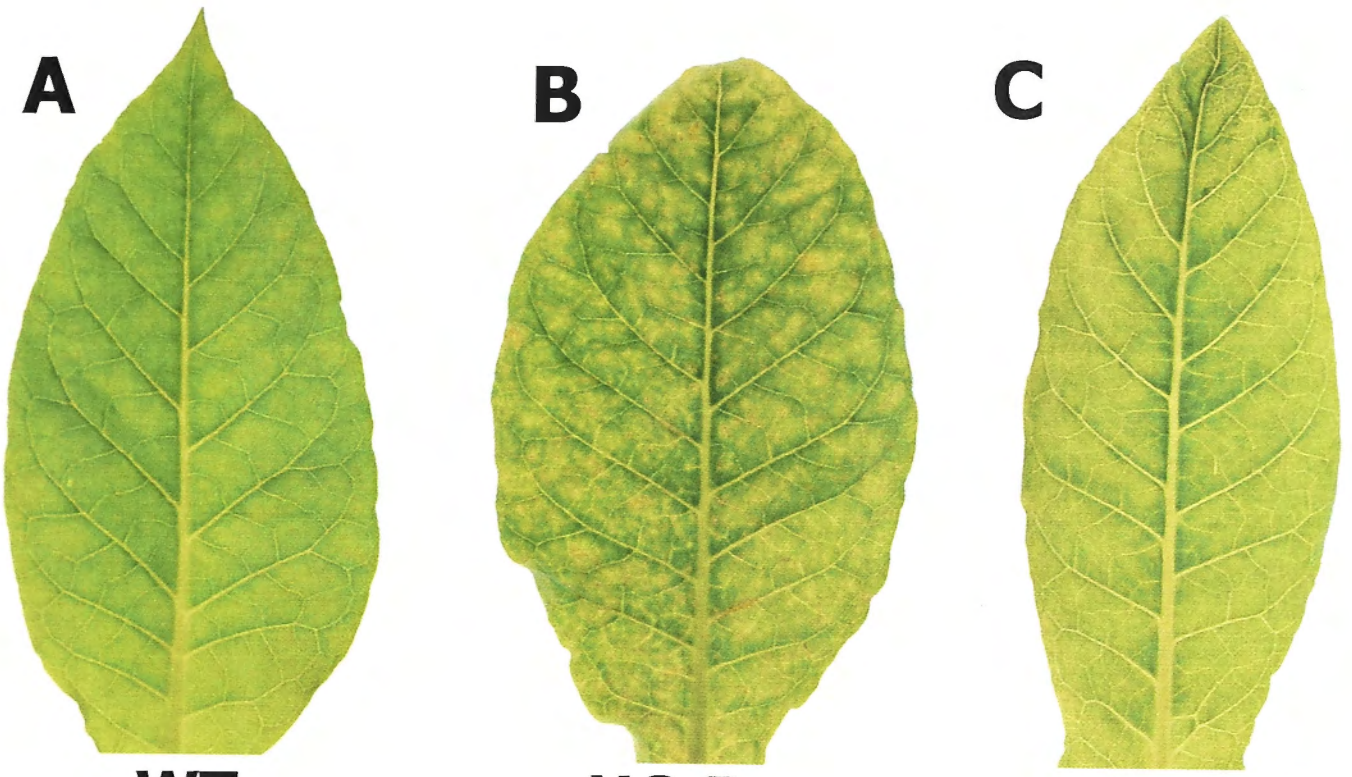
C HC-Pro expressing plants inoculated with PVY-D followed by 55N

D Plants carrying S+AS transgene and an empty vector backbone; inoculated with PVY-D

E S+AS and HC-Pro expressing plants; inoculated with PVY-D

F Plants carrying hpRNA transgene and an empty vector backbone; inoculated with PVY-D

G hpRNA and HC-Pro expressing plants; inoculated with PVY-D


A
B
C
WT
HC-Pro
HC-Pro
PVY-D>55N
55N
PVY-D>55N
D
E
S+AS x Vec
S+AS x HC-Pro
F
G
Hp x Vec
Hp x HC-Pro

PVY-D resistance. All segregants that inherited either S+AS or hpRNA transgene but not the HC-Pro transgene were immune to the virus (Fig.2.6).

To test whether *in planta* expression of HC-Pro could interfere with cross-protection, four sets of eight plants expressing only HC-Pro were inoculated with each of the PVY^{O} strains. All of these plants were subsequently challenged with the necrotic PVY 55N strain (Fig. 2.5 B, C). Four weeks after inoculation with 55N the plants were examined for necrotic symptoms. None of the tested plants exhibited significant necrosis suggesting that all tested plants were effectively cross-protected (Fig. 2.6).

These experiments show that cross-protection, hpRNA- and S+AS-mediated virus protection all provide a similar level of resistance against a suite of PVY strains. However, a difference in the response to the *in planta* expressed HC-Pro suggests that mechanisms mediating cross-protection and transgene-mediated protection may differ.

2.3.2 The effect of simultaneous or sequential challenge with different viral strains on S+AS and hpRNA mediated protection

Although S+AS and hpRNA both displayed good protection against a homologous PVY-D strain and variant PVY strains, it is possible that the efficacy of the protection could be reduced if two different viral strains simultaneously challenged the same plant. To investigate this possibility, wild type, S+AS and hpRNA plants were challenged with a combination of PVY-D and 55N strains.

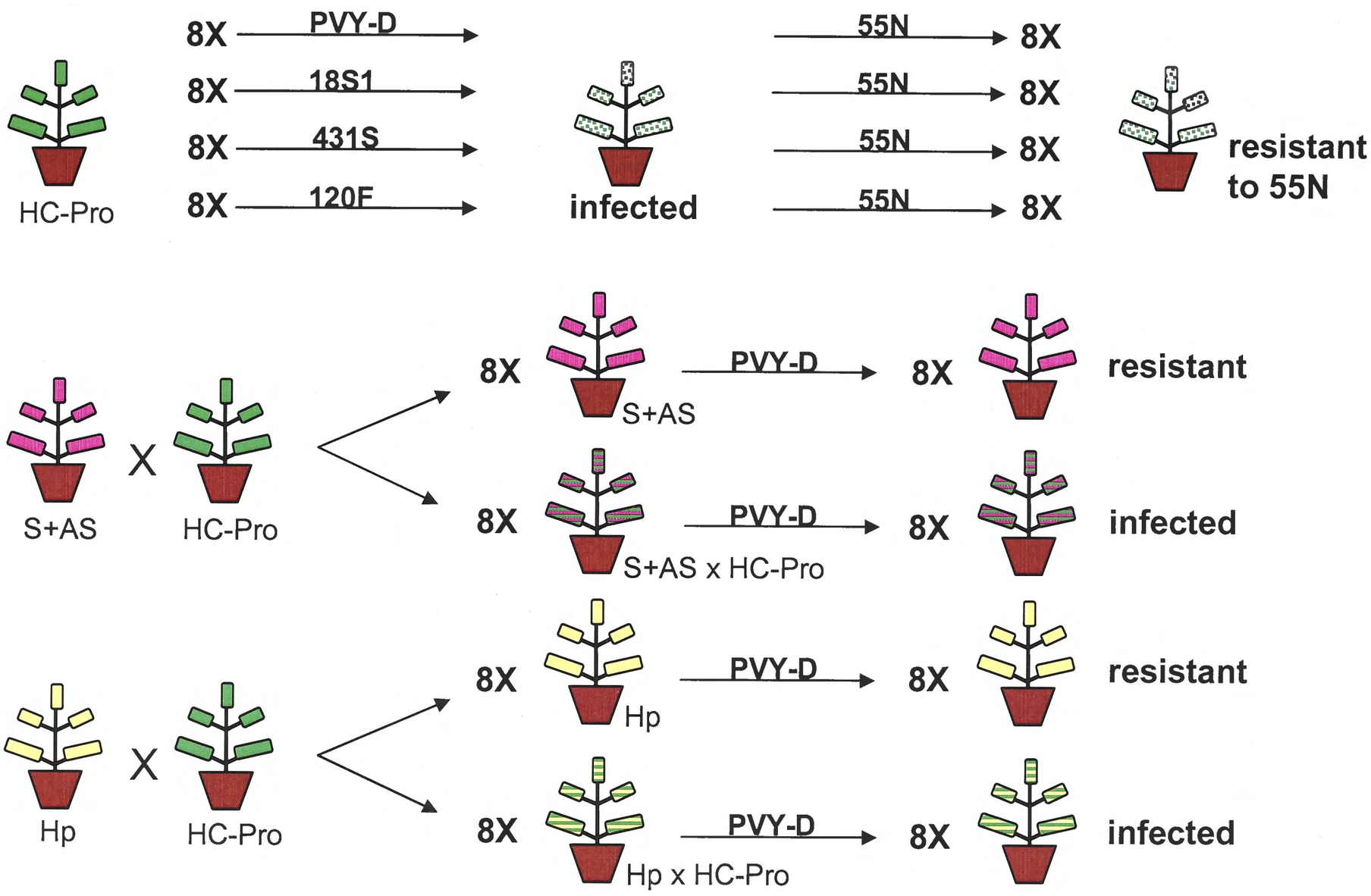


Figure 2.6: Summary outlining the effect of HC-Pro on cross protection and transgene-mediated protection

Eight non-transgenic, eight S+AS and eight hpRNA plants were challenged simultaneously with D and 55N, or with either D or 55N, and then three weeks later with the reciprocal virus. Neither S+AS nor hpRNA plants showed the chlorotic symptoms of D infection or the necrotic symptoms of 55N infection, or detectable levels of either virus after inoculation with any of the inoculation regimes (Fig. 2.7). Non-transgenic plants inoculated with 55N alone, 55N followed by D, or 55N and D together, showed severe necrotic symptoms and had high levels of virus. Non-transgenic plants inoculated with D alone or D followed by 55N showed chlorotic symptoms and had high virus levels, measured by ELISA. These findings show that both S+AS and hpRNA transgenes give good protection against a range of PVY strains when applied singly, sequentially or in combination.

2.3.3 Relationship between PVY isolates and the transgenes

As S+AS and hpRNA plants displayed a robust resistance to a suite of divergent PVY strains, I wanted to establish what homology existed between the resistance-mediating transgene and the challenging virus. The NIa gene of each PVY variant strain was amplified by polymerase chain reaction, cloned and sequenced. The overall sequence identity between NIa from PVY D and its counterparts in the variant strains was ~84%. Each variant strain had at least two blocks of continuous 21bp identity with the transgene sequence (Fig. 2.8). These blocks represent targets for 21nt siRNAs produced from the transgenes. In contrast, the transgenes did not provide protection against alfalfa mosaic virus (AMV) which had less than 30% overall sequence identity, and no 20nt blocks of identity, with the PVY-derived transgenes (Fig. 2.8, Fig. 2.9). This shows that both S+AS- and the hpRNA-mediated

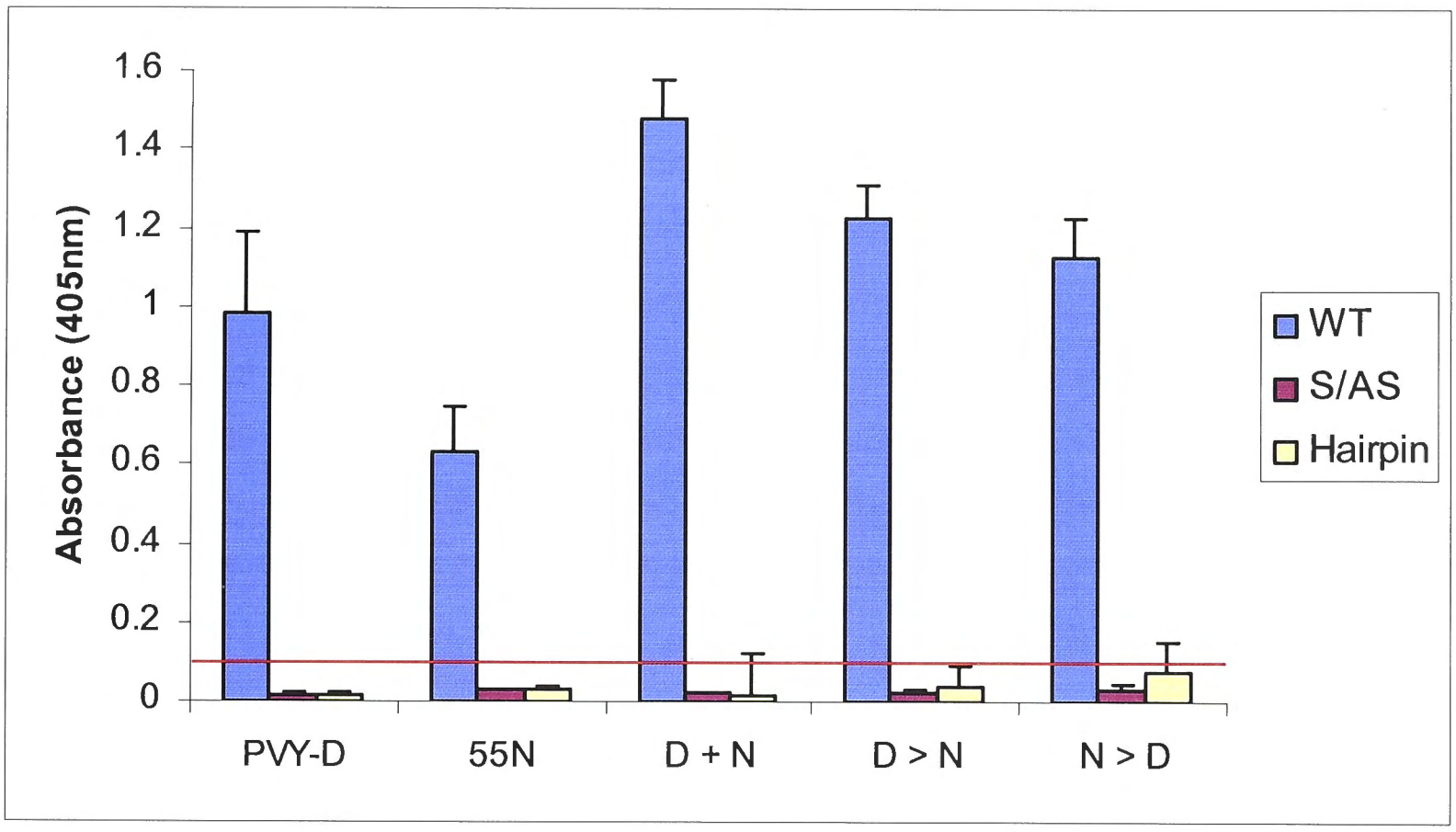


Figure 2.7: Simultaneous or sequential inoculation of WT, S+AS and Hairpin plants.

Ten wild type, S+AS and hairpin plants were manually inoculated with single virus (PVY-D or 55N), two viruses at the same time (D + N), with PVY-D followed by 55N (D > N) or the inverse (N > D). Virus levels were measured by ELISA. The columns represent median values while error bars are derived from standard error values. The horizontal line marks 405nm absorbance that equals to 2× the average absorbance value for uninfected tissue samples. Tissue samples are considered to be infected with a virus if their 405nm absorbance value is above the level marked with the horizontal line.

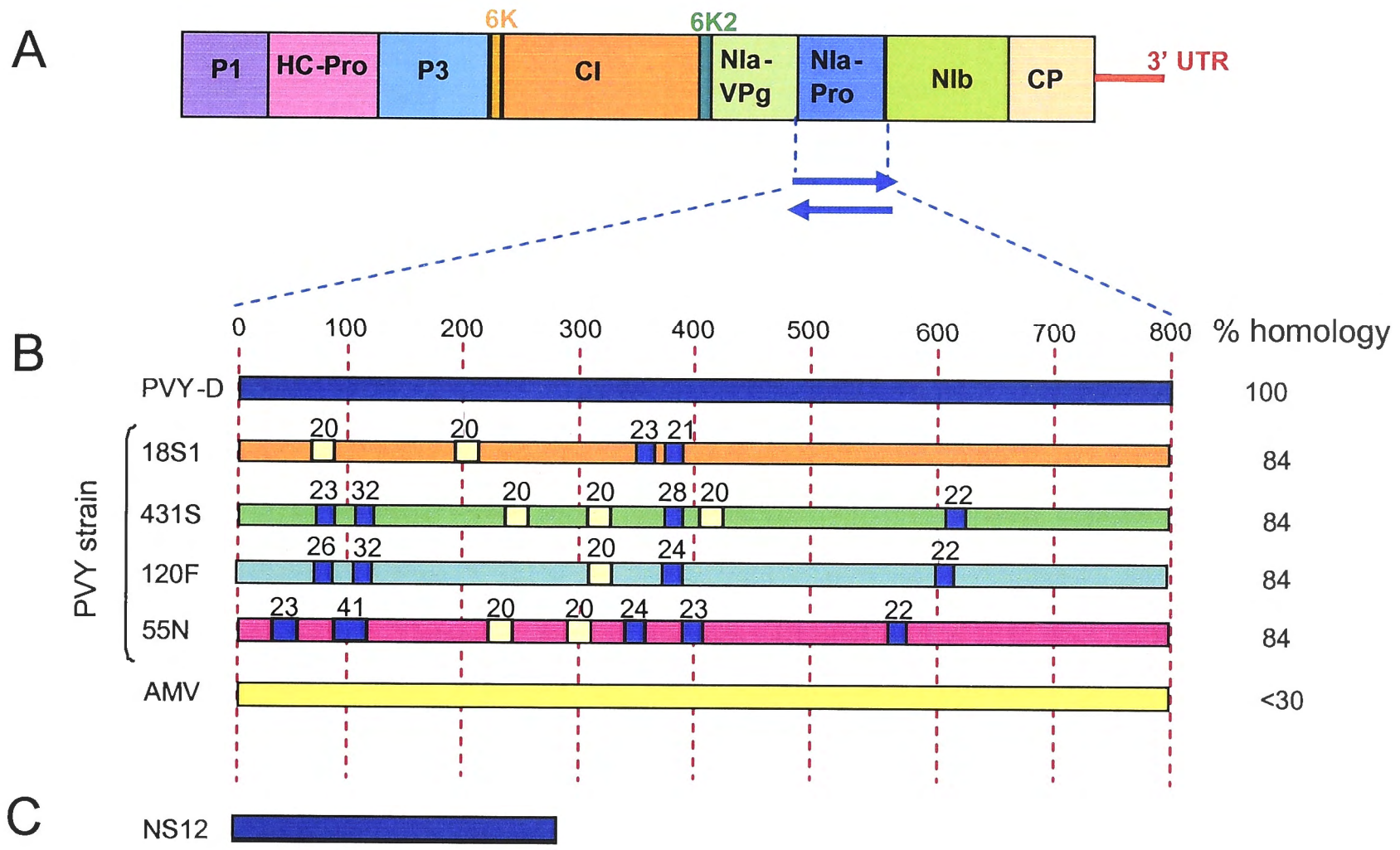


Figure 2.8: Schematic representation of PVY derived transgenes and sequence comparison with four divergent PYV strains and AMV.

A. A representation of the PVY genome. Blue arrows denote the area of the genome from which the S+AS and hairpin transgenes were derived.

B. Sequence comparison between the transgenes and the four PVY strains. Blue rectangles denote sequence identity blocks of 21bp or more. The size of each identity block is noted above the blue rectangles. Yellow rectangles denote blocks of 20bp sequence identity.

C. Schematic representation of the transgene present in the NS12 plant line.

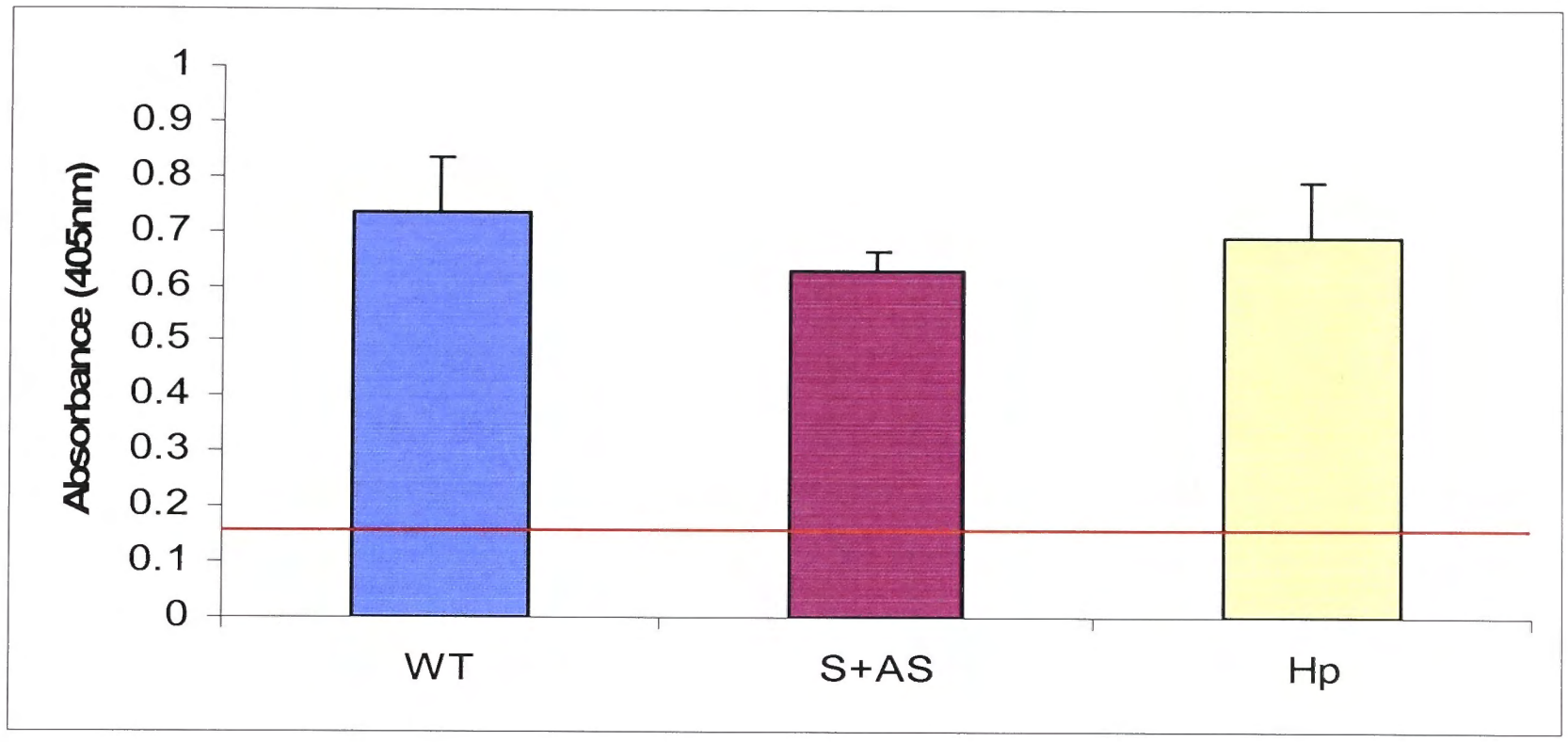


Figure 2.9: Comparison of viral levels in wild type, S+AS and hairpin plants inoculated with AMV.

Ten wild type, S+AS and hairpin plants were inoculated with AMV. Virus levels were measured by ELISA three weeks post inoculation. The columns represent median values while error bars are derived from standard error values. The horizontal line marks 405nm absorbance that equals to 2× the average absorbance value for uninfected tissue samples. Tissue samples are considered to be infected with a virus if their 405nm absorbance value is above the level marked with the horizontal line.

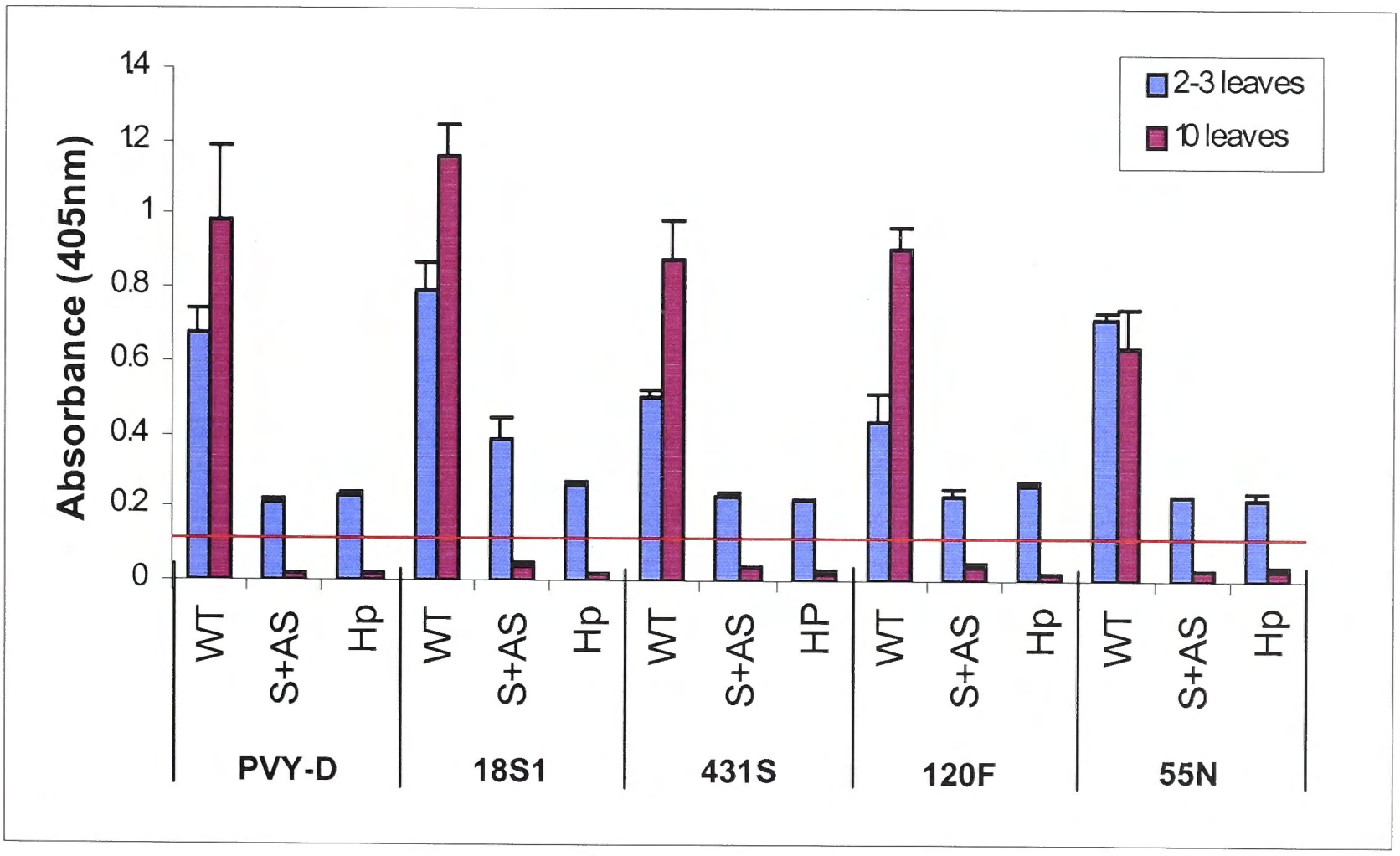


Figure 2.10: Comparison of virus levels in small (2-3 leaves) and medium (10 leaves) wild type, S+AS and hairpin plants.

Ten wild type, S+AS and hairpin plants were inoculated with each of PVY strains. Virus levels were measured by ELISA two weeks post inoculation. The columns represent median values while error bars are derived from standard error values. The horizontal line marks 405nm absorbance that equals to 2× the average absorbance value for uninfected tissue samples. Tissue samples are considered to be infected with a virus if their 405nm absorbance value is above the level marked with the horizontal line.

protection are equally effective against viruses containing only a few target blocks of 21nt identity.

2.3.4 Plant age and the number of 21nt sequence identity blocks affect the efficacy of transgene mediated protection

To test whether the age of the plant had an effect on the efficiency of the two different forms of transgene-mediated protection, very young (2-3 leaves) S+AS and hpRNA plants were challenged with PVY strain variants. ELISA readings taken two weeks after inoculation revealed that neither the S+AS nor the hpRNA plants were initially immune to the variants (Fig. 2.10). Each virus strain, including the PVY-D which has 100% identity with the transgene, was able to replicate to levels detectable by ELISA in the small S+AS and hpRNA plants, although these levels were much lower than in the wild type plants. However, 4 weeks later, tests on the youngest fully expanded leaf of each plant showed that both the S+AS and hpRNA plants (now at ~10 leaf stage) had eliminated or restricted the virus to a point below the level of detection.

The distribution of each virus in the S+AS and hpRNA plants was further investigated. Extracts from the inoculated leaf, and each of the four leaves above it, were analysed by ELISA (Fig. 2.11). In the wild-type plants, PVY-D and the other variants (data not shown) were present at similar levels in all tested leaves, except in the youngest, not fully expanded leaf (leaf 5). Virus levels detected in this type of leaf were consistently lower than the levels detected in the fully expanded leaves. In the transgenic plants, the strength of protection against the virus strains was ranked: D>N>431S>120F>18S1. The strongest protection was against PVY-D, which was

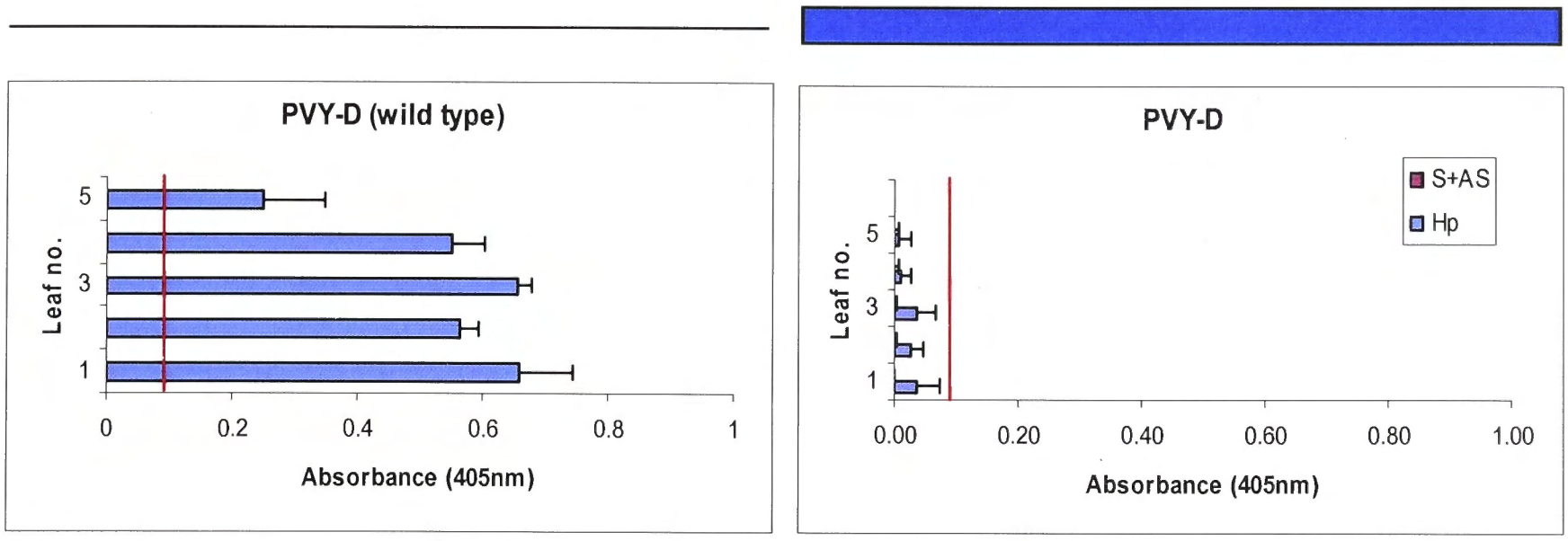


Figure 2.11: The evolution of transgene mediated virus resistance in young plants. Virus levels in eight S+AS plants that were inoculated at 2-3-leaf stage were measured by ELISA six weeks post-inoculation (4 weeks after the initial ELISA testing was done). The leaf numbered as 1 corresponds the inoculated leaf. Leaves numbered form 2 to 5 correspond to leaves progressively further up the plant. The line above each graph represents the homology shared between the transgene and the inoculated virus. Blue squares represent homology blocks of ≥21nt while yellow squares represent homology blocks of 20nt.

not detected in any of the tested leaves. These included the inoculated leaf, suggesting that virus had actually been eliminated over the 4 weeks. A low level of PVY-N was detected in the inoculated leaf (leaf 1), but not in subsequent leaves. Strains 120F and 431S had similar profiles, with a moderate level of virus in the inoculated leaves but with little or no virus in the higher leaves. Strain 18S1 had moderate levels of virus in both the inoculated leaf and the leaf above it, but very low virus levels in the subsequent two leaves.

The trends observed in this experiment can also be viewed in terms of sequence identity blocks shared between the transgene and the challenging virus. For example, the S+AS transgene provided very good protection against the PVY-N strain with which it shares 5 blocks identity of at least 21nt (Fig. 2.8, Fig. 2.11). The protection against the 18S1 strain, which shares only two 21nt sequence identity blocks with the transgene, was much less effective. Interestingly, both viral strains have the same overall homology with the transgene (84%). This observation indicates that the effectiveness of protection correlates not with the degree of overall homology between the transgene and the virus, but with the number of sequence identity blocks spanning at least 21nt (Fig. 2.12). It is possible that siRNAs derived from the transgene can only efficiently mediate cleavage of the viral RNA if the viral genome contains a match of $\geq$ 21nt in length.

The distribution of each virus strain in the hpRNA plants was also investigated. Interestingly, 6 weeks post inoculation hpRNA plants had almost entirely eliminated the inoculated virus from the tested leaves. Although a small amount of virus was detected in plants inoculated with 18S1 and 55N strains, the hpRNA transgene

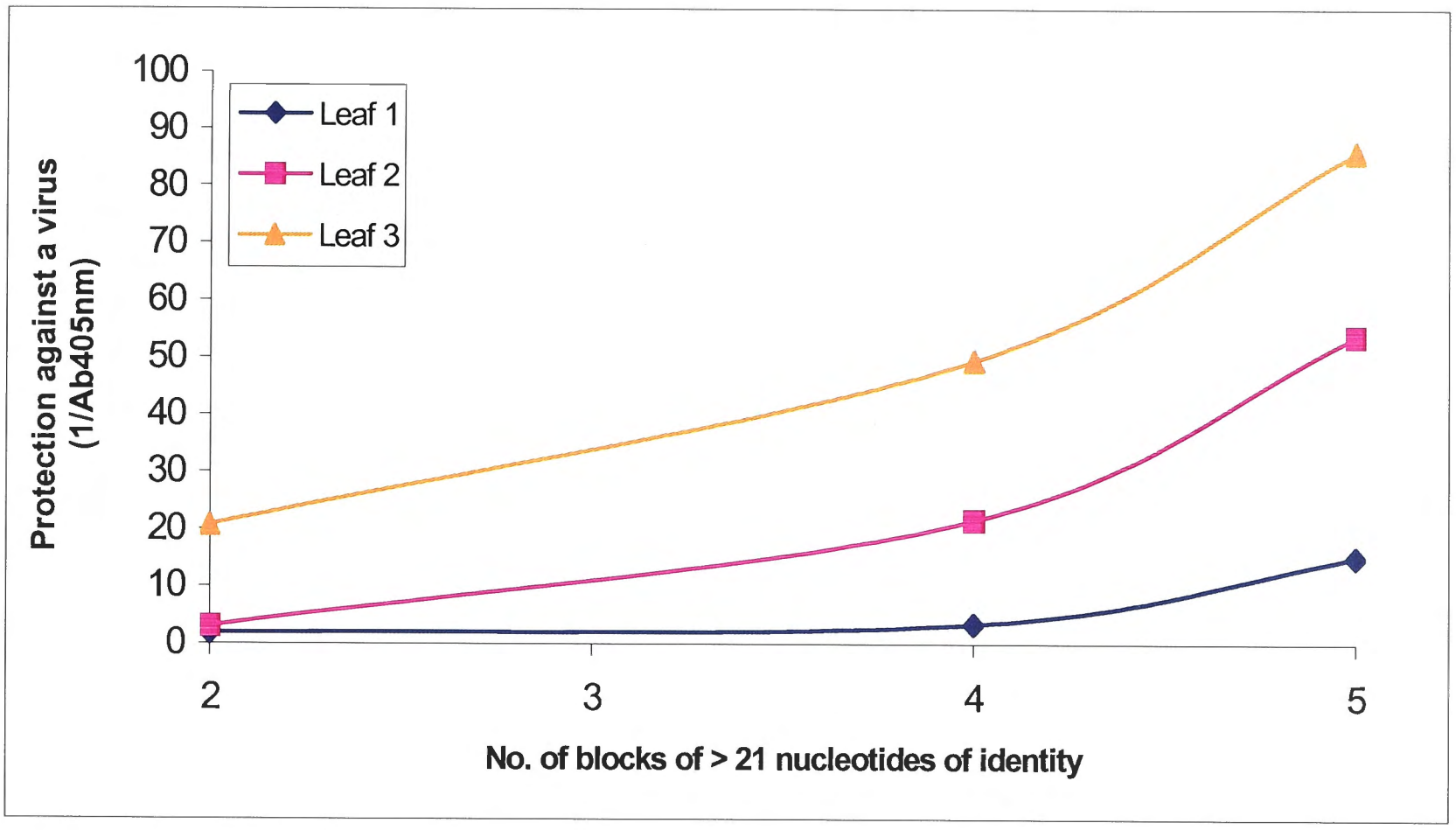


Figure 2.12: Inverse correlation between virus accumulation and the number of ≥ 21bp sequence identity blocks shared between the virus and the S+AS transgene.

Values on the axis labelled "protection against virus" were obtained by taking the inverse value of Ab405nm. High values of 1/Ab405nm indicate low viral titre and good protection. All trends were derived from data presented in Fig. 2.11. 18S1 readings were used to generate plots for a virus containing two sequence identity blocks. Plots for the virus containing 4 sequence identity blocks were derived from the averaged data for the 431S and 120F strains. ELISA readings from the 55N strain were used to generate plots for a virus containing 5 sequence identity blocks.

appeared to be equally effective in mediating rapid elimination of all viral strains (Fig. 2.11).

2.3.5 Protection against viruses sharing less than 21bp continuous identity with the transgene

As the specificity unit for RNAi in animals was originally described as 21nt (Zamore *et al.* 2000) the correlation between the number of 21nt identity blocks and the degree of virus resistance is not unexpected. To further test this correlation I used the NS12 plant line which expresses 280bp sense and antisense RNAs derived from the 5' terminal of the original PVY D Nia transgene. PVY strains 431S, 120F, 55N and 18S1 share 2, 2, 2 and 0 blocks of at least 21nt identity with NS12, respectively (Fig. 2.8). If one or more 21nt siRNAs are required for protection, then the NS12 plants will protect against PVY strains, D, 55N, 431S and 120F but not against 18S1. In contrast to this prediction, the NS12 plants appeared to be as well protected against 18S1 as against the other strains (Fig. 2.13). The longest block of sequence identity between the NS12 transgene and the NIa of 18S1 are two 20nt blocks: GAAGCTAAATCGCTCATGAGaGG (where "a" in the transgene mismatches with a "G" in virus) and ATAGCGAACCACCATTTGTTtCT (where "t" in the transgene mismatches with a "C" in virus).

2.3.6 The efficiency of S+AS and hairpin RNA mediated silencing in a non-viral system

As hpRNA and S+AS transgenes were both able to induce PTGS of homologous viral genes it was expected that the same would apply to other silencing systems. This hypothesis was partly addressed by a study which examined progeny plants resulting from a cross between plants expressing GUS RNA in a hairpin conformation (plant

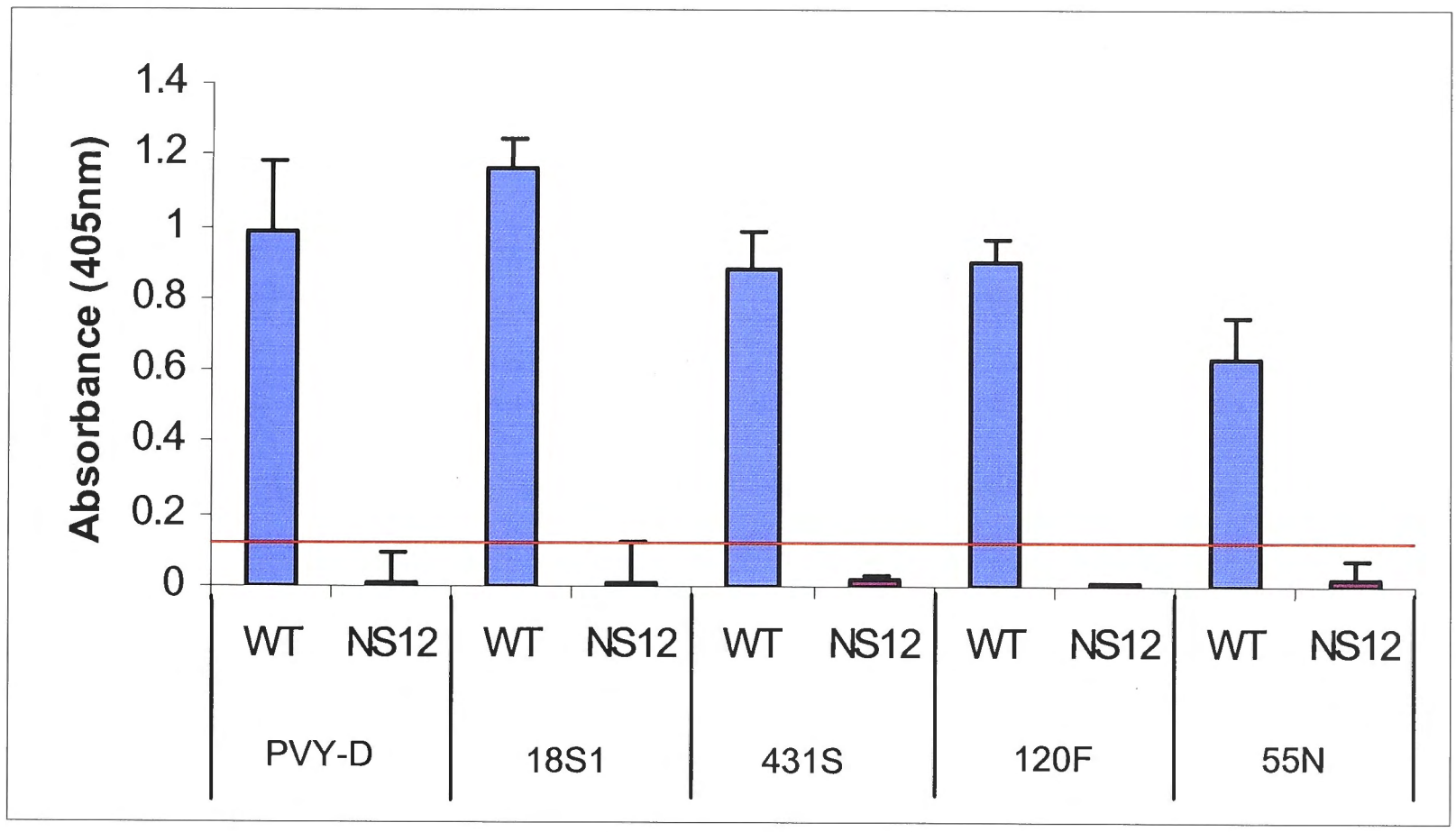


Figure 2.13: Comparison of virus levels in wild type and NS12 plants.
Ten wild type (WT) and NS12 plants were inoculated with each of the viral stains. Virus levels were measured by ELISA at three weeks post-inoculation. The columns represent median values while error bars are derived from standard error values. The horizontal line marks 405nm absorbance that equals to 2× the average absorbance value for uninfected tissue samples. Tissue samples are considered to be infected with a virus if their 405nm absorbance value is above the level marked with the horizontal line.

line 8.7) and plants expressing an active β-glucuronidase (GUS) protein (Wesley *et al.* 2001). This study found that plants containing both transgene constructs do not show any GUS activity and accumulate siRNAs derived from the GUS transcript. Furthermore, the silencing efficiency of the GUS hairpin construct was close to 100% (Wesley *et al.* 2001). These observations suggest that the GUS hpRNA transgene induces post-transcriptional silencing of the previously active GUS transgene.

To test whether a combination of sense and antisense (S+AS) transgenes could induce GUS silencing I crossed plants expressing active GUS protein with four different plant lines (NS40-1,2,3 and 4 plant lines) expressing GUS RNA in an antisense direction. Twelve progeny plants from each cross were stained for GUS activity (Fig. 2.14). All tested plants showed significant GUS activity indicating that the presence of GUS S and AS transgenes does not induce GUS silencing.

These finding suggests that S+AS and hairpin silencing constructs do operate in distinct ways. It is not clear why the S+AS construct is so efficient in inducing PTGS of PVY and yet so ineffective in inducing PTGS of the GUS transgene. It is possible that viral proteins, such as viral RdRP, contribute to generation of dsRNA from separately synthesised sense and antisense transcripts. Alternatively, PVY RNA may be more sensitive to PTGS than GUS mRNA, or may itself play a part at inducing PTGS. To investigate these possibilities we infected previously analysed GUS S+AS plants with PVY-D. The presence of the virus was confirmed by ELISA two weeks post inoculation and the plants were re-tested for GUS activity the following week (3 weeks post inoculation). Five out of 48 tested plants showed a degree of GUS silencing, however some plants appeared to have a mosaic silencing pattern (Fig.

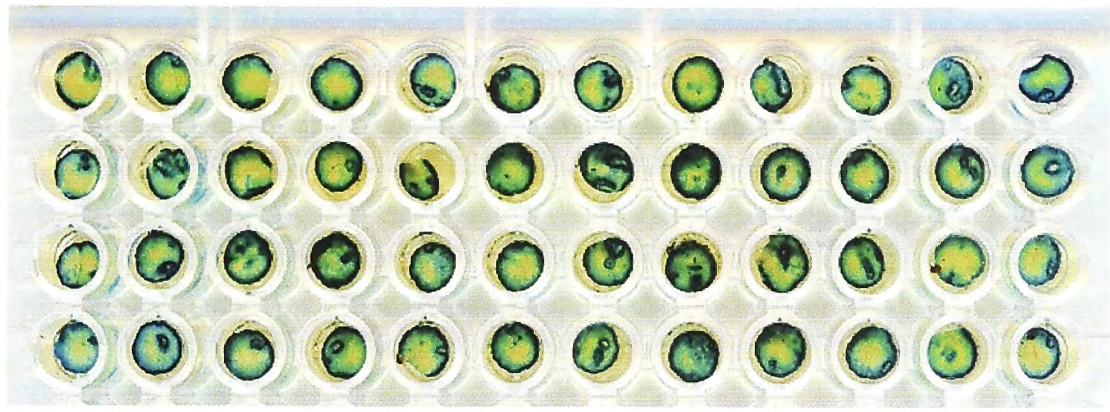
Figure 2.14: Effect of PVY infection on GUS expression in GUS S+AS plants.

A Twelve plants from 4 different GUS S+AS lines were stained for GUS expression. All tested plants were GUS +ve.

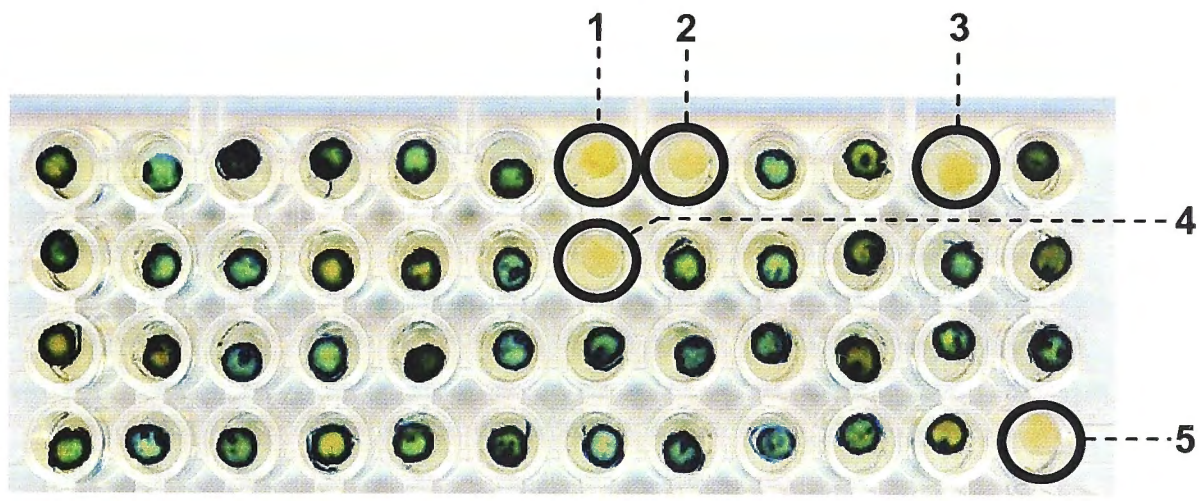
B The same set of plants was tested for GUS activity three weeks post PVY inoculation. Five out of 48 tested plants were GUS-silenced

C Multiple samples were taken from each of the GUS-silenced plants and stained for GUS activity.

A


NS40-1
NS40-2
NS40-3
NS40-4

B


NS40-1
NS40-2
NS40-3
NS40-4
1
2
3
4
5

C


1
2
3
4
5

2.14). These findings indicate that the presence of the virus can facilitate the onset of PTGS of an unrelated gene; however this appears to be a stochastic and low frequency event.

2.4 Discussion

In this chapter I compared qualitative characteristics of cross-protection and transgene (S+AS and hpRNA) mediated protection for a range of Australian PVY isolates. The observation that plants expressing S+AS or hpRNA transgenes accumulate siRNAs (Fig. 2.1) indicates that in these plants, pre-established PTGS mediates virus protection. It has been suggested that cross protection operates on a similar principle, with PTGS being established in the wake of primary viral infection (Ratcliff *et al.* 1999). If this were the case, then PTGS established in response to the primary infection would be able to protect the plant from subsequent infection by any virus that shares sufficient sequence identity with the original virus. However, transgenes that operate via PTGS are known to offer more specific protection, limited to viral strains that share significant sequence identity with the transgene, whereas cross-protection tends to be broader (Wang *et al.* 1987, Wang *et al.* 1991, Namba *et al.* 1992, Pang *et al.* 1992, Pang *et al.* 1993, Maki-Valkama *et al.* 2000). One possible explanation for this difference in the breadth of protection is that in cross-protection, siRNAs could be derived from the entire viral genome while in transgene-mediated protection, siRNAs can only be derived from the relatively short transgene sequence. Consequently, in the case of cross-protection, there would be a more diverse pool of siRNAs that could be used to silence any other invading virus. Alternatively, it is possible that cross-protection operates via a distinct mechanism that may share some, or very little, similarity with PTGS.

As the first step in investigating these possibilities, I compared the breadth of protection offered by the two transgenes (S+AS and hpRNA) and cross-protection in the PVY-based experimental system. The breadth of protection offered by the two transgenes was tested by the inoculation of plants expressing S+AS and hpRNA transgenes (derived from PVY-D) with a suite of divergent PVY stains. The breadth of cross-protection was tested by first infecting wild-type plants with a PVY^{O} variant and, following the establishment of infection, inoculating with a PVY-55N necrotic variant. Cross-protection was evaluated by testing for the presence of necrotic symptoms typical of the 55N variant. Based on the results described in this chapter, all three forms of protection (S+AS and hpRNA transgenes and cross-protection) were similarly effective in protecting tobacco plants against tested PVY isolates. Therefore, in this experimental system, I could not distinguish between mechanisms involved in S+AS or hpRNA mediated protection and cross-protection based solely on the breadth of protection each of these methods offers.

I extended the comparison between protection mediated by S+AS and hpRNA transgenes and cross-protection by investigating the effect of HC-Pro, a known suppressor of gene silencing, on these forms of protection. The experimental system used to evaluate the effect of HC-Pro on transgene-mediated protection was established by crossing plants expressing either S+AS or hpRNA transgenes with plants that carried HC-Pro derived from TEV. Progeny plants carrying HC-Pro together with S+AS or hpRNA transgene were then inoculated with PVY-D. As expected, all plants expressing HC-Pro together with the S+AS or hpRNA transgene

were now susceptible to PVY, indicating that HC-Pro was able to suppress the PTGS-based protection that these transgenes offer.

The effect of HC-Pro on cross protection was examined by infecting plants expressing HC-Pro with a PVY^{O} strain and then, following the establishment of infection, inoculating with the 55N necrotic strain. The effectiveness of cross-protection was evaluated by testing for presence of necrosis typical of 55N infection. Surprisingly, none of the tested plants displayed any necrotic symptoms indicating that cross-protection was not affected by the expression of HC-Pro.

Initially I considered the possibility that this unexpected finding was due to the silencing of the *in planta* expressed HC-Pro by siRNAs derived from the corresponding gene from a PVY^{O} strain which was used for primary infection. Sequence comparisons revealed that *in planta* expressed HC-Pro, which was derived from TEV, and PVY^{O} HC-Pro share only one 20nt sequence identity block. Although this limited sequence identity appears to be sufficient for the establishment of silencing (see section 2.3.5 and further discussion) a number of other factors makes this scenario unlikely. To begin with, it is difficult to explain how PVY^{O} could initiate silencing when HC-Pro protein is already present in the cytoplasm of cells that are infected with PVY^{O}. HC-Pro does not prevent the formation of siRNAs, but is thought to suppress PTGS by inhibiting the RISC complex which incorporates siRNAs and uses them to guide cleavage of the cognate single stranded RNA target (Silhavy & Burgyan 2004). Therefore, if siRNAs derived from PVY^{O} were to be used to silence *in planta* expressed HC-Pro they would need to enter the nucleus, combine with a nuclear-based RISC complex and guide cleavage of HC-Pro pre-mRNA (Fig.

2.15). If this were the case, the cytoplasmic pool of HC-Pro protein would eventually be depleted and cross-protection could proceed unhindered. However, this is an unlikely scenario, firstly because all currently available experimental data indicate that the RISC complex is only present in the cytoplasm and secondly because it has been shown that during viral infection, siRNAs do not enter the nucleus (reviewed in Silhavy & Burgyan 2004, Denti *et al.* 2004). Therefore, we are left with the possibility that cross-protection operates via a mechanism distinct from PTGS. Although it has been suggested before that cross-protection might operate on a protein level, another possibility is that cross protection operates via an alternative gene silencing pathway. Some support for this hypothesis can be found in a recent study which showed that viral suppressors of gene silencing can have a different effect on silencing induced by an inverted repeat to that involving micro RNAs (Dunoyer *et al.* 2004). It would be interesting to further test this hypothesis by comparing the effect of other known viral suppressors of gene silencing on transgene mediated silencing and cross-protection.

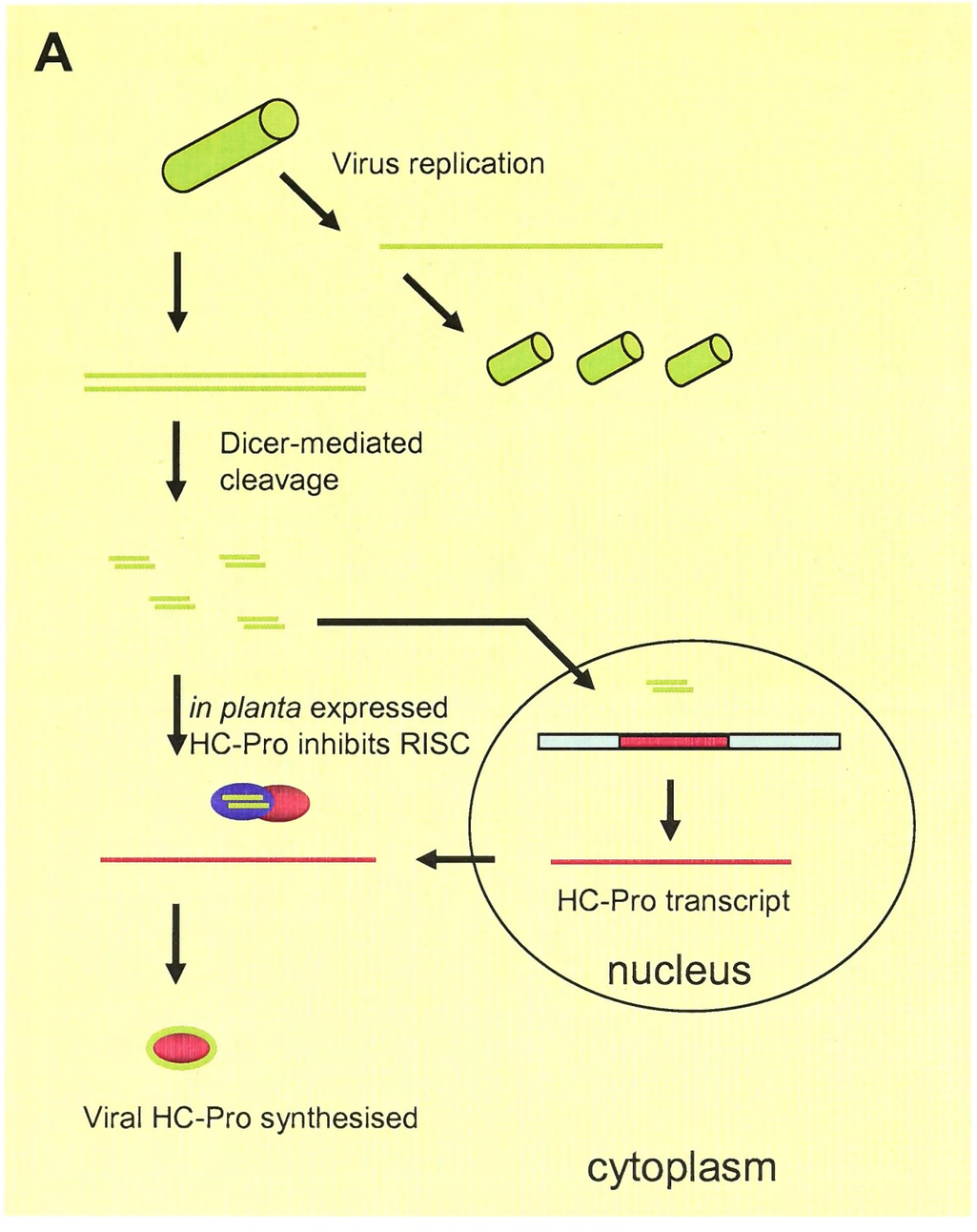
Further investigation focused on qualitative comparison of virus protection offered by S+AS or hpRNA transgenes. The first set of experiments involved inoculation of medium sized plants (8-10 leaves) expressing S+AS and hpRNA with a suite of PVY strains. I found that both S+AS and hpRNA transgenes offered very good protection against all PVY strains tested. In addition, I was not able to detect any difference in quality or efficiency of protection offered by these two transgenes. This was somewhat surprising since I expected that the S+AS transgene combination, which produced only a small amount of siRNAs prior to viral infection, would not offer as

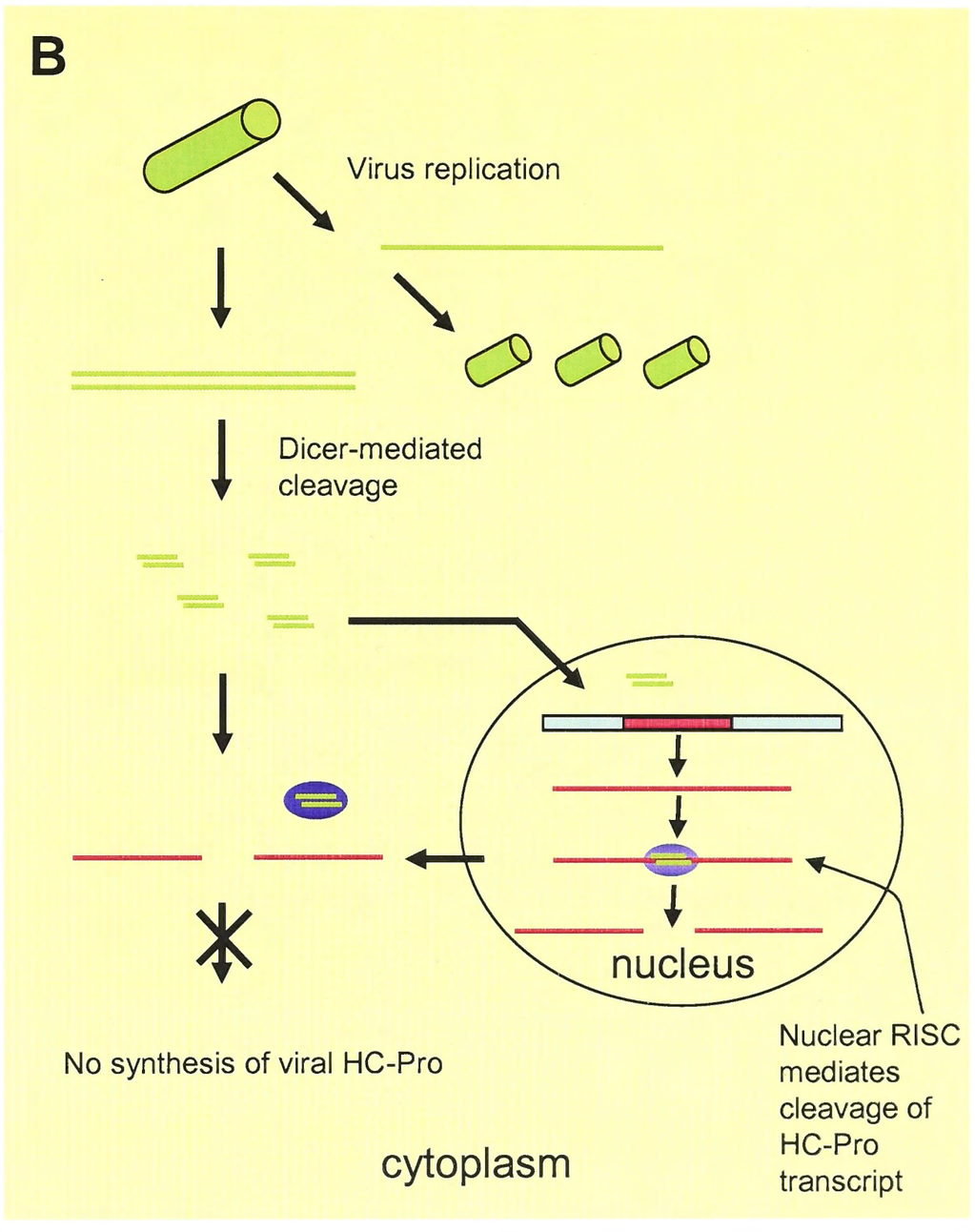
Figure 2.15: An outline of events leading to possible silencing of HC-Pro.

During PVY replication some dsRNA is generated and cleaved into siRNAs by DICER. At the same time HC-Pro mRNA is produced from the transgene and HC-Pro protein is made (A).

PVY-derived HC-Pro and i*n planta* expressed HC-Pro (derived from Tobacco Etch Virus) share one 20bp sequence identity block. However, since HC-Pro protein is already present in the plant interferes with RISC, HC-Pro mRNA is not degraded.

It is possible that some of the PVY-derived siRNAs can enter nucleus (B). Once in the nucleus these siRNAs could combine with nuclear RISC and mediate degradation of HC-Pro pre-mRNA. As a result, HC-Pro mRNA would be depleted and HC-Pro protein would no longer be produced (B).


A
Virus replication
Dicer-mediated
cleavage
in planta expressed
HC-Pro inhibits RISC
HC-Pro transcript
nucleus
Viral HC-Pro synthesised
cytoplasm


B
Virus replication
Dicer-mediated
cleavage
nucleus
No synthesis of viral HC-Pro
Nuclear RISC
mediates
cleavage of
HC-Pro
transcript
cytoplasm

good protection as the hpRNA transgene, which induced accumulation of a high level of siRNAs prior to viral infection.

It has been previously reported that the efficiency of transgene-mediated gene silencing may be dependent on plant age (Pang *et al.* 1996, Kalantidis *et al.* 2002). In order to investigate this phenomenon further and extend the comparison between S+AS and hpRNA transgenes, I repeated the previous experiment using very young plants (2-3 leaves) expressing S+AS or hpRNA transgenes. Two weeks post inoculation both S+AS and hpRNA plants accumulated virus to levels which, although lower than those observed in wild type plants, were above the "non-infected" threshold. When the same seedlings were re-tested four weeks later (a total of six weeks after the inoculation), I found that regardless of the viral strain, hpRNA plants had almost entirely eliminated the virus, while S+AS plants were gradually recovering and reducing viral levels.

Although these experiments demonstrated that both protection mediated by S+AS and hpRNA transgenes was less efficient during early development, I was not able to determine the reason. It is possible that RNAi and related mechanisms play an important role in the control of endogenous gene expression in young seedlings and consequently, the cellular machinery is not as accessible for silencing events mediated by transgenes. Alternatively, silencing pathways may simply not be active and so the cellular machinery is unavailable during early development. As the plants age, silencing appears to be activated and the transgenes begin to mediate elimination of the accumulated virus. Therefore, it appears that S+AS and hpRNA transgenes are equally efficient in preventing a viral infection but differ in their ability to combat

already accumulated virus. Perhaps the relatively large level of siRNAs in plants carrying hpRNA transgene leads to more rapid elimination of virus of virus when the defence machinery is available. In contrast, the S+AS transgene produces a relatively small amount of siRNAs which could become rate limiting for virus elimination.

Alternatively, the two transgenes may use distinct branches of the silencing pathway and therefore require a different subset of cellular machinery. For example, co-suppression, unlike silencing mediated by hpRNA transgenes, requires the activity of RNA dependent RNA Polymerase (RdRP) and probably involves an amplification step (Dalmay *et al.* 2000, Mourrain *et al.* 2000, Beclin *et al.* 2002, Vaistij *et al.* 2002). It is possible that the S+AS transgenes operate via a co-suppression branch of the pathway, and therefore require RdRP activity. Keeping this hypothesis in mind, it is interesting to note that the rate of recovery of S+AS seedlings closely correlated with the number of ≥ 21 nucleotide sequence identity blocks shared between the virus and the transgene. S+AS seedlings eliminated the 55N viral strain, which has five such blocks, more quickly than viral strains 120F and 431S, which have four ≥ 21nt sequence identity blocks. The recovery process was slowest in seedlings inoculated with the strain 18S1, which has only two ≥ 21nt sequence identity blocks.

If we accept the possibility that silencing mediated by S+AS transgenes involves an amplification step, then the following model may be proposed. Initially only a small proportion (out of a relatively small pool) of siRNAs derived from the S+AS transgenes have an exact match with the target virus. However, these siRNAs can act as primers for amplification from the viral RNA. Consequently, dsRNA and many siRNAs with complete identity to the virus are synthesised and can now be used to

guide cleavage of viral RNA. The rate at which this process proceeds is likely to be dependent on the initial number of functional primers, which is determined by the number of ≥ 21nt sequence identity blocks shared between the transgene and the challenging virus. This scenario is analogous to the human immune response where initially only a small number of antibodies recognise an antigen and cannot effectively eliminate it. However, eventually the immune response is activated, appropriate antibodies are amplified, and the antigen is eliminated.

The possibility that the silencing mechanism used by the S+AS transgenes may involve an amplification process raised two additional questions, namely:

1. What was the minimum number of 'primer' sites required for the establishment of silencing; and
2. What length of continuous sequence identity between siRNA and RNA template was required for a priming event?

The S+AS plant line NS12, expressing 280bp sense and antisense RNAs derived from the 5' terminal of the original S+AS transgenes, was identified as a suitable model for the exploration of these questions. NS12 plants were inoculated with PVY strains 431S, 120F, 55N and 18S1 that shared 2, 2, 2 and 0 blocks of ≥ 21nt identity with the NS12 line, respectively. Furthermore, strain 18S1 shared only two 20nt sequence identity blocks, and no 19nt sequence identity blocks with the NS12 transgene. As predicted by previous experiments this plant line was immune to the 431S, 120F and 55N strains. However, somewhat surprisingly, it was also immune to the 18S1 strain. This result suggests that if siRNAs, complementary to the virus act as primers, then the 20nt sequence identity blocks were sufficient to allow priming,

generation of dsRNA and successful elimination of the virus before it could accumulate to detectable levels.

Previous studies have attempted to determine the minimum sequence identity, between an siRNA and its target RNA, that is required for induction of PTGS. Studies using *in vitro* experimental systems have found that the minimum required sequence identity ranges from 19 to 21 nucleotides (Elbashir *et al.* 2001c, Martinez *et al.* 2002) On the other hand, studies conducted *in vivo* suggest the required sequence identity ranges from 21 to 23 nucleotides (Boutla *et al.* 2001, Thomas *et al.* 2001,). In the experimental system I used, the minimum required sequence identity was 20nt, which is shorter than the lengths indicated by previous *in vivo* studies. It would be interesting to design S+AS transgenes that share even shorter sequence identity blocks with the challenging virus and define the minimum sequence identity required for effective PTGS in this experimental system.

The experiments discussed so far demonstrate that S+AS transgenes can effectively induce PTGS targeted at viral RNA. To investigate whether S+AS transgenes could be as effective in inducing PTGS in other systems I crossed plants expressing a GUS transgene in the sense orientation with plants expressing the same transgene in the antisense orientation, effectively creating a GUS S+AS transgene. All medium sized plants (8-10 leaves) carrying the GUS S+AS constructs continued to accumulate GUS mRNA and produce an active GUS protein, suggesting that PTGS targeted at GUS mRNA was not induced. Interestingly, when these GUS S+AS plants were inoculated with PVY-D, GUS silencing was induced in ~ 10% of plants. The silencing event was stochastic and did not appear to correlate with plant age or virus

levels. In some plants, silencing was mosaic, while in others it appeared to affect the entire plant.

It is not clear why the presence of a virus induced GUS silencing. One possibility is that a viral factor, perhaps viral RdRP, was able to initiate silencing. Alternatively, the presence of a virus could up-regulate the detection system used by the plant to identify sequences with potential to form double stranded RNA molecules. These options could be further explored by investigating whether silencing correlates with the presence of a virus inside a particular cell. In the experimental system I used it was not possible to simultaneously test for PVY presence and GUS silencing in a specific area of the plant. This problem could be overcome by infecting tobacco plants with cucumber mosaic virus, which causes yellowing of infected areas and leaves green islands in the areas where the virus is not present. The relationship between the viral presence and onset of GUS silencing could then be investigated by staining yellow and green areas for GUS expression.

Chapter 3

An investigation of the systemic spread of gene silencing

3.1 Introduction

3.1.1 Aims

In previous studies describing the systemic spread of PTGS three types of tissues acted as sources of the silencing signal. These included: (i) plant lines that displayed spontaneous silencing due to the presence of complex transgene loci (Palauqui *et al.* 1997, Sonoda & Nishiguchi 2000, Crete *et al.* 2001), (ii) tissues in which PTGS had been induced by biolistic introduction of multiple transgene copies (Palauqui & Balzergue 1999, Crete *et al.* 2001) and (iii) the tissues in which PTGS had been induced by agro-infiltration (Voinnet & Baulcombe 1997, Voinnet *et al.* 1998). It is still not known whether plant lines carrying S+AS or hpRNA transgenes are able to produce a graft-transmissible silencing signal which can induce PTGS in tissues where the gene of interest was previously active. This chapter aims to determine whether S+AS and hpRNA transgenes generate such a signal. In addition, I explore how grafting methods and plant age can affect the capacity of grafted tissues to process the silencing signal and to establish gene silencing.

3.1.2 Systemic spread of gene silencing

One of the intriguing features of gene silencing is that it is not cell-autonomous – it can be induced locally and then spread systemically into distant parts of a plant. This phenomenon was first noticed by research groups studying transgenic plants in which the transgenes were developmentally silenced (Boerjan *et al.* 1994, Palauqui *et al.* 1996). In these plants, transgene silencing was induced spontaneously in the lower leaves of young seedlings and spread in the direction of phloem flow. In the lower leaves, where the silencing was initiated, the silenced phenotype was usually confined

to the vascular tissue. In the successive leaves the area of the silenced tissue increased until all the newly developed leaves displayed the silenced phenotype. Interestingly, when the silencing was initiated in very young seedlings the spread of silencing was fast and often the whole plant was affected. In contrast, when the silencing was initiated in an older plant, the spread was very slow and uneven resulting in a variegated phenotype (Palauqui *et al.* 1996).

3.1.3 Characteristics of the silencing signal

These initial reports of the systemic spread of PTGS were followed by a number of studies aiming to characterise the patterns of spread of the silencing signal and its molecular nature. Some of the findings from these studies are summarised below:

1. The silencing signal appears to travel from cell to cell via plasmodesmata. The observation that the stomatal guard cells, which do not have plasmodesmal connections, escape the silencing that affects all neighbouring tissues supports this hypothesis (Voinnet *et al.* 1998).

2. The pattern of systemic spread of the silencing signal resembles the phloem transport of dyes and viruses (Roberts *et al.* 1997). The suggestion that the silencing signal is transported through phloem is also supported by the observation that spread of silencing initially affects vasculature and, from there, progresses into mesophyll cells (Voinnet *et al.* 1998).

3. The silencing signal can be transported over long distances and through graft junctions (Palauqui *et al.* 1997, Voinnet *et al.* 1998).

4. Most reports suggest that the silencing signal only travels upward from the site of silencing (Palauqui *et al.* 1997, Palauqui & Vaucheret 1998, Crete *et al.* 2001). However, a downward spread of the signal has also been reported (Sonoda & Nishiguchi 2000).

5. The spread of gene silencing is always sequence specific, suggesting that the signal has a nucleic acid component, most probably RNA. The exact composition of the silencing signal is not known. It has been suggested that siRNAs, either in a protein complex or on their own, could operate as a mobile silencing signal (Klahre *et al.* 2002). Alternatively, the signal could be composed of a longer dsRNA, or a protein-tagged target RNA (Mlotshwa *et al.* 2002).

3.1.4 Experimental methods used to study systemic spread of gene silencing

The experimental methods previously used to study the spread of PTGS include agro-infiltration (Voinnet & Baulcombe 1997) or biolistic introduction of sequences that induce gene silencing (Palauqui & Balzergue 1999), and grafting of silenced plants in combination with tissues in which the target gene is still active (Palauqui *et al.* 1997) (Table 3.1).

A disadvantage of the agro-infiltration and biolistic methods is that they lead to the introduction of an unknown number of transgene copies into a small area of the plant. Although these methods clearly lead to the establishment and the spread of PTGS, the use of pathogenic organisms and/or introduction of numerous transgene copies can

Table 3.1: Experimental methods used to study the systemic spread of PTGS. (adapted from Mlotshwa *et al.* 2002)

System	Method	Advantages / Disadvantages
Grafting	• Use a part of a plant in which a gene has been silenced as a source of signal. • Graft the silenced plant in combination with tissues in which the same gene is still active. • Allow vascular connection to form between the two tissues. • Monitor the establishment of silencing in the previously active tissue.	• Able to control the source of silencing signal • No pathogenic organisms required • Labor intensive and slow
Agro-infiltration	• Engineer *Agrobacterium* to contain T-DNA sequences homologous to the target transgene. • Use a syringe to infiltrate one or more lower leaves with the engineered *Agrobacterium*. • Silencing is established in the infiltrated area • Monitor the establishment of silencing in upper non-infiltrated leaves.	• Versatile • Many different T-DNA constructs can be assayed quickly • Introduction of a pathogenic organism may complicate the interpretation of results
Biolistics	• Engineer plasmid to contain DNA sequences homologous to the target transgene • Bombard one or more lower leaves with the plasmid-coated gold particles. • Silencing is established in the bombarded area. • Monitor establishment of silencing in upper non-bombarded leaves.	• Versatile and fast • No pathogenic organisms required • Expensive

complicate the analysis. In contrast, a grafting approach allows the use of well-characterised tissue, containing single transgene copies, as a source of the silencing signal.

3.1.5 Grafting

Grafting involves joining parts of different plants in such a manner that allows these tissues to unite and continue growth as one plant. The lower part of the graft is termed the rootstock, while the top section is called the scion.

The origins of grafting can be traced back to ancient times. Evidence suggests that grafting was known to the Chinese as early as 1000 B.C., while detailed descriptions of grafting methods can be found in writings dating back to the Roman Empire. During the Renaissance period grafting was used to propagate many new plant varieties imported from foreign countries (Hartman& Kester 1975).

In horticulture, grafting is used to alter the characteristics of fruit cultivars. For example, fruit growers often graft the desired fruit cultivar onto a rootstock that causes dwarfing and makes harvesting easier. In most cases the rootstock influences the quality of the fruit produced by the scion. For example, when sweet orange (*Citrus sinesis*) seedlings are used as a rootstock for orange trees the fruits are thin-skinned and juicy (Hartman *et al.* 1981). Although in most cases the rootstock influences the scion, sometimes the scion can also influence the growth habit of the rootstock. For example, in apple seedlings the scion can control the morphology of the root system developed by the rootstock (Hartman & Kester 1975).

3.1.6 Grafting methods

More than 120 different grafting techniques have been previously described. However, in investigations of the systemic spread of gene silencing three main methods have been used (Crete *et al.* 2001) (Fig. 3.1):

- Reciprocal grafts, made by exchanging the bottom and the top sections of the plants. A V-shaped section is cut out of the rootstock stem and an appropriately shaped scion stem is inserted into the gap (Fig. 3.1, A).
- Plug grafts made by exchanging transverse cylinders of stem cut from pairs of plants (Fig. 3.1, B)
- Top grafts made by inserting a very young shoot into the stems of a mature rootstock (Fig. 3.1, C)

Regardless of the grafting method, a contact between the cambium tissues of the rootstock and scion is crucial for successful establishment of the graft.

The formation of the graft union can be divided into the three main phases. During the first phase cambial regions of the scion and the rootstock tissues start producing callus tissue (parenchyma cells) (Fig. 3.2). The second phase is marked by the differentiation of the callus cells into new cambium cells. The new cambium cells establish a contact between the original cambial layers of the rootstock and the scion (Fig. 3.2). In the final phase the new cambium differentiates into xylem and phloem, thus establishing a vascular connection between the rootstock and the scion (Hartman & Kester 1975, Esau 1977).

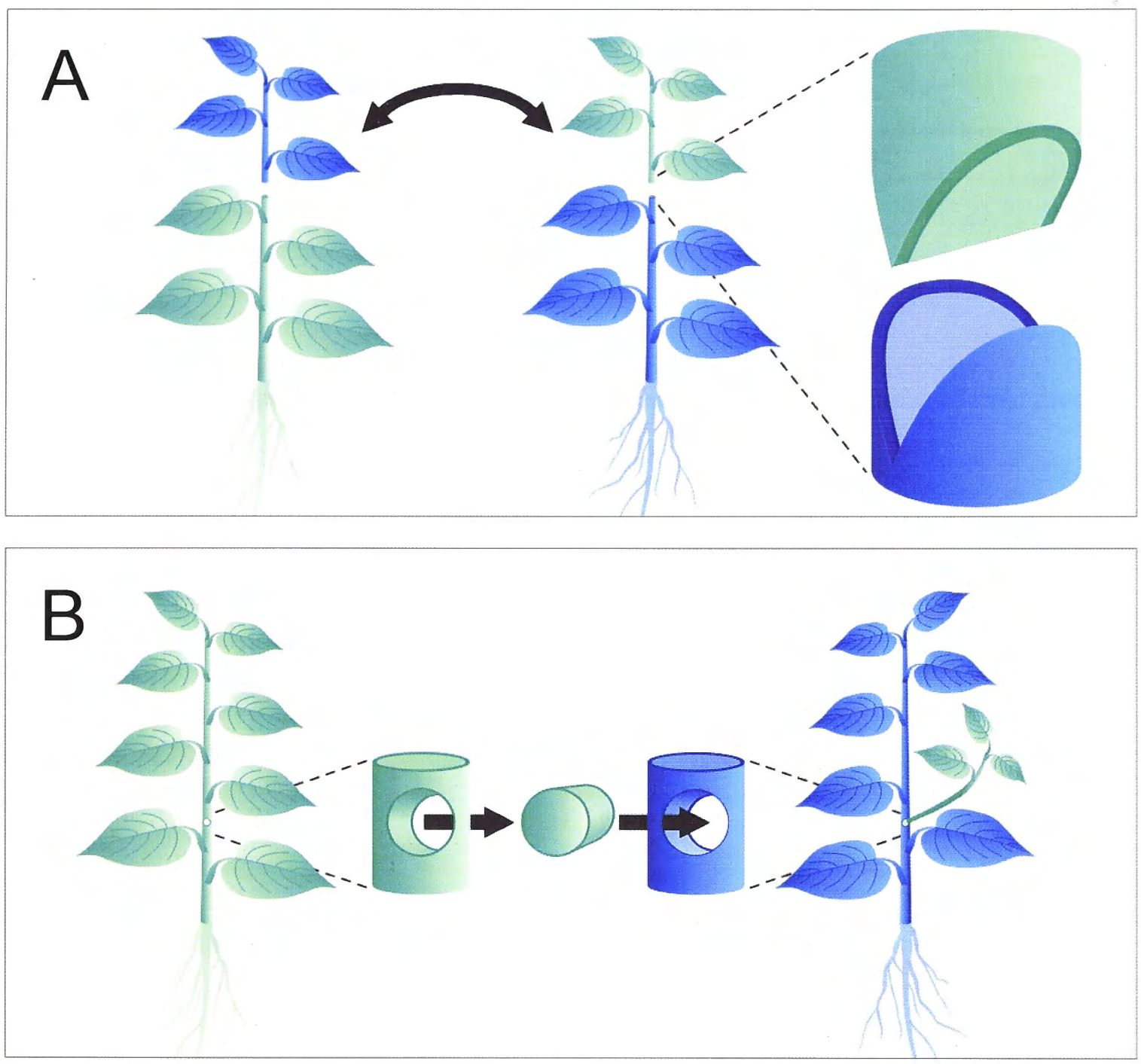


Figure 3.1: Grafting methods used in studies that investigated the systemic spread of gene silencing.

A. Reciprocal grafting

B. Plug grafting

C. Top grafting

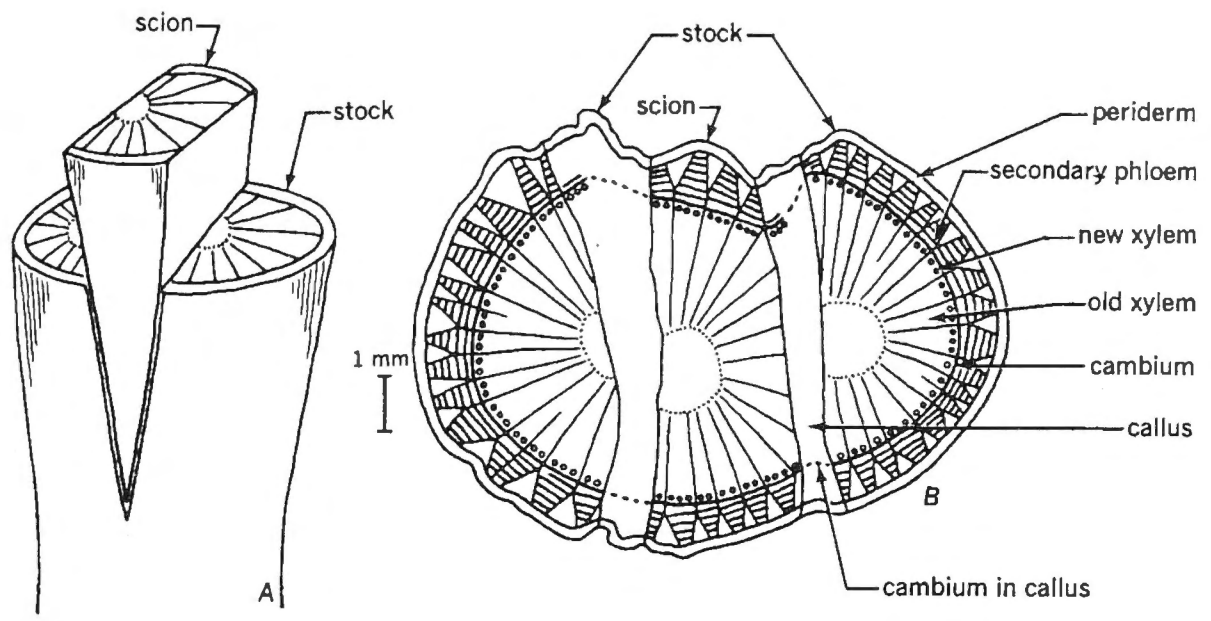


Figure 3.2: Illustration of the stock and scion junction. (reproduced from Esau 1977)

3.1.7 Signaling across graft junctions

Little is currently known about the molecular interactions between rootstocks and scions. However, recent evidence indicates that proteins and nucleic acids produced in the rootstock can travel across graft junctions and influence the development of the scion.

Heterografting experiments, using pumpkin as a rootstock and cucumber as a scion, demonstrated that proteins and RNA species can be trafficked over long distances and across graft junctions (Xoconostle-Cazares *et al.* 1999). Analysis of sap collected from the cucumber scion revealed that the pumpkin CmPP16 protein and its transcript had accumulated in the scion. Moreover, the sap derived from the cucumber scion was devoid of the cucumber homologue of CmPP16 indicating that proteins and/or transcripts imported from the pumpkin rootstock could influence transcription or translation within the scion.

Grafting experiments performed by Kim *et al.* (2001) provide a striking example of the influence that a rootstock can exert on a scion. The authors used a tomato line

carrying the *Mouse ears* (*Me*) mutation associated with altered leaf shape as a rootstock. The scions were derived from the *Xa* tomato line that carries a mutation resulting in yellow leaves with wild-type morphology. After the graft was established, the new leaves emerging from the *Xa* scion had the morphology associated with *Me* mutation. Examination of the shoot apex under scanning electron microscope revealed that these changes occurred very early in the development. This observation indicates that the transcript associated with the *Me* mutation could access the meristem tissue and influence the leaf development.

Palauqui *et al.* (1997) were first to demonstrate that rootstocks silenced for nitrate reductase were able to induce silencing of this gene in scions in which the nitrate reductase transgene was originally active. The spread of silencing was not affected by the introduction of a 30cm leaf-less stem between the graft and scion, indicating that the silencing signal could travel over long distances. The spread of PTGS across graft junctions was subsequently demonstrated for a number of other genes including β-glucuronidase (GUS), green fluorescent protein (GFP), chitinase and a coat protein gene derived from sweet potato feathery mottle poty-virus (Palauqui *et al.* 1997, Voinnet *et al.* 1998, Sonoda & Nishiguchi 2000, Crete *et al.* 2001).

3.1.8 Experimental system

3.1.8.1 PVY-resistant plant lines and PVY strains

PVY resistant plant lines expressing S+AS and hpRNA constructs were described in Section 2.1.3.2. Analysis of RNA species in hpRNA plants revealed that these plants accumulate high levels of PVY-specific siRNAs prior to viral infection (Fig. 2.1).

Much lower levels of siRNAs were detected in S+AS plants indicating that in these plants, PTGS was only weakly induced prior to viral inoculation (Fig. 2.1).

The experiments described in Chapter 2 demonstrated that both S+AS and hpRNA constructs could mediate effective protection against a suite of PVY strains. A differentiating feature between these two constructs was their ability to protect very young seedlings. Although neither construct was able to completely prevent infection, the virus was more efficiently eliminated from the seedlings expressing the hpRNA construct.

In this chapter, I investigate whether S+AS and hpRNA tissues are able to generate a graft transmissible silencing signal which could establish PTGS-based protection in unprotected tissues. The spread of silencing was tested by challenging the grafted tissues with PVY-D or 55N. These PVY strains have been described in Section 2.1.3.3.

3.1.8.2. GUS silenced and GUS expressing plant lines

The 6b5 plant line carrying the bacterial-derived GUS gene under the control of the 35S promoter was donated by Dr Hervé Vaucheret (INRA, Versailles Cedex, France). Young 6b5 seedlings have a low level of GUS activity but within 1 month of germination, the GUS gene becomes silenced. This process is even more rapid in plants homozygous for the 6b5 construct. In GUS-silenced plants, the 6b5 transgene is highly transcribed. However the levels of GUS mRNA are very low, suggesting that this plant line exhibits post-transcriptional silencing (Elmayan & Vaucheret 1996). In addition, 6b5 plants can generate a graft-transmissible silencing signal. When used as

rootstocks in previous grafting experiments 6b5 plants were able to induce GUS silencing in scions that had high GUS activity prior to grafting (Palauqui *et al.* 1997). Following discussions with Dr Hervé Vaucheret I included 6b5 plants into this study as an experimental control.

The plant line expressing the GUS silencing construct was generated and evaluated by Dominikus Akhadi. The silencing construct consists of the full-length GUS gene in sense and anti-sense orientations, separated by a pyruvate orthophosphate dikinase (PDK) intron. Transcription of this construct results in the production of GUS RNA in a hairpin conformation (GUS Hp). Introduction of the GUS Hp construct into a GUS expressing background (GUS x GUS Hp) results in GUS silencing in all progeny plants carrying both transgenes. Accumulation of high levels of GUS-derived siRNAs in GUS Hp and GUS x GUS Hp plants suggests that silencing operates via a PTGS mechanism (Wesley *et al.* 2001).

In this chapter I use grafting experiments to investigate whether tissues expressing the GUS Hp construct can induce GUS silencing in tissues that had a high level of GUS activity prior to grafting. Plants with a high level of GUS activity that were used in these experiments were generated by Lisa Molvig (CSIRO Plant Industry, ACT, Australia). These plants carry a bacterial-derived GUS gene under control of the 35S promoter and a phosphinothricin acetyl transferase (BAR) selectable marker, also under control of the 35S promoter.

3.2 Materials and methods

3.2.1 Plants and viruses

S+AS and HpRNA tobacco plants lines have been previously described (Waterhouse *et al.* 1998, Smith *et al.* 2000). Plants expressing GUS were generated by Lisa Molvig (CSIRO Plant Industry, Australia). GUS silenced plants, containing the GUS transgene and the GUS hairpin silencing construct, have been previously described (Wesley *et al.* 2001). The 6b5 GUS-silenced line was provided by Dr Hervé Vaucheret (INRA, Versailles Cedex, France). PVY strains (D and 55N) were provided by Dr John Thomas (QDPI, Indooroopilly, Australia). PVY strains were propagated in tobacco. Glasshouse temperatures were 25°C during the day and 20°C in the night.

3.2.2 Reciprocal grafting

N. tabacum plants were grown to a height of ~40cm. Scions were prepared by cutting of the top section of the stem (~6cm), removing most of the leaves and shaping the bottom of the stem into a V-shape (Fig. 3.1, A). The rootstocks were prepared from the bottom portion of the plant by cutting a V-shaped section out of the stem (Fig. 3.1, A). Scion and rootstock portions were joined together by wrapping Parafilm© (USA) around the stem. The plants were covered with plastic bags to maintain humidity while the grafts were being established. The plastic bags were opened four days after grafting and removed a week later.

3.2.3 Top grafting

N. tabacum plants used as rootstocks were grown to a height of ~40cm. Each rootstock was prepared for grafting by removing the top section of the plant (~8cm)

and cutting a small V-shaped section out of the cambium of the remaining stem (Fig. 3.1 C). Plants used as scions were grown until they had 3-4 leaves. Such plants were prepared for grafting by cutting the roots off, shaping the stem into a "V" and removing all but one leaf. Rootstocks and scions were joined together by wrapping Parafilm© (USA) around the stem. Small plastic bags were placed over the scions to maintain humidity while the grafts were being established. The plastic bags were opened one week after grafting and completely removed after an additional week.

3.2.4 Evaluation of systemic spread of PVY silencing

The grafts were inoculated with a PVY isolate ~3 weeks post grafting. Inoculation was performed as described in section 2.2.3. Three weeks after the inoculation, the rootstocks and scions were either scored for the presence of viral symptoms (Fig. 2.2, A, B and C, Fig. 3.3) or analysed for PVY presence by ELISA. ELISA-based analysis was performed as described in section 2.2.4.

3.2.5 Evaluation of systemic spread of GUS silencing

Eight weeks after grafting, leaf-disc samples were taken from one of the leaves below the grafting point and the four leaves above the grafting point. The leaf discs were stained for GUS expression as described in section 2.2.5.

3.3 Results

3.3.1 Systemic spread of PVY silencing in reciprocal grafts

Although several reports describe the transmission of gene silencing from silenced rootstock tissues into previously active scion tissues, the systemic spread of the

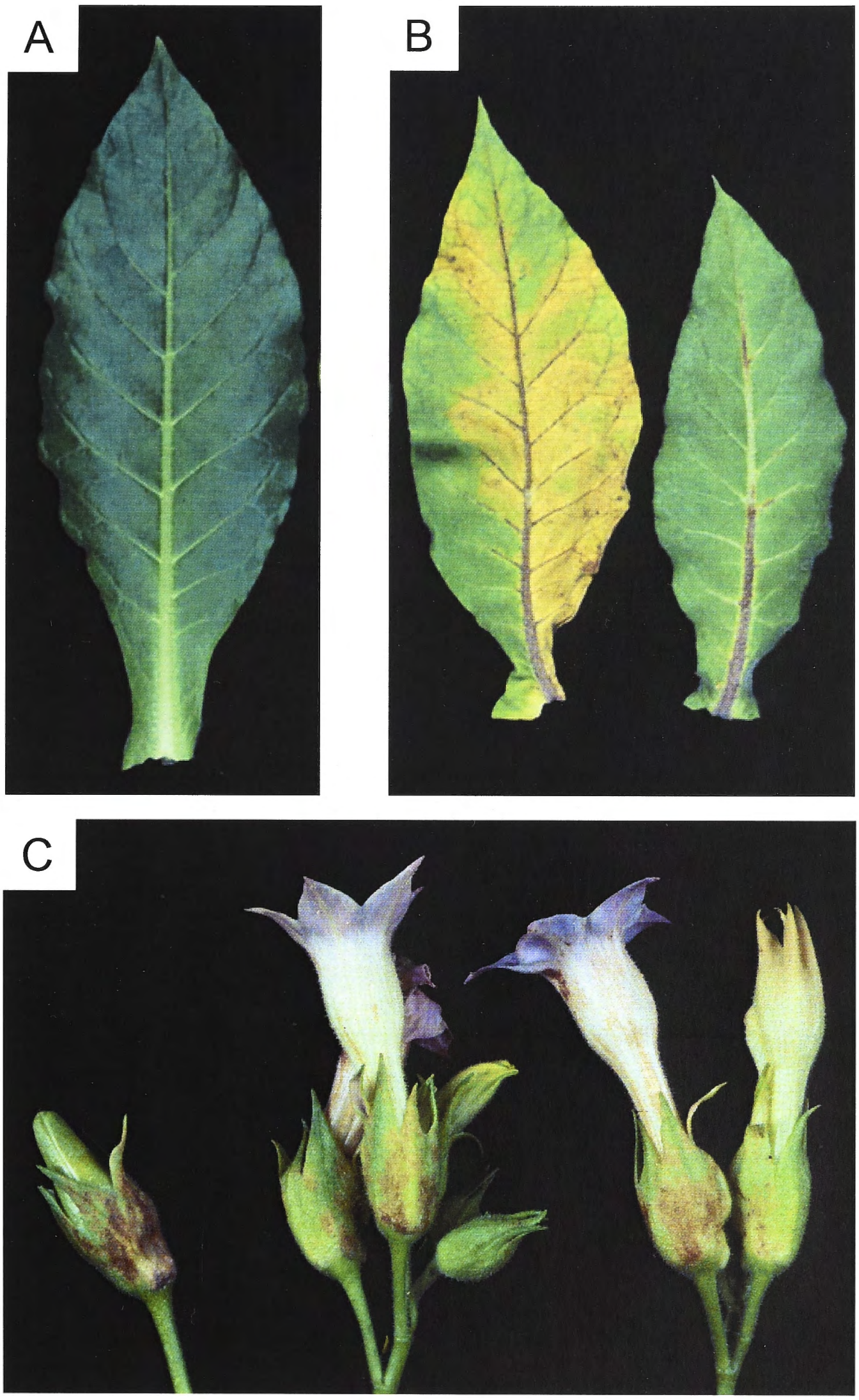


Fig. 3.3: Symptoms associated with PVY-55N infection.

A. An uninfected leaf

B. Leaves displaying typical PVY-55N symptoms

C. Flowers and pods showing PVY-55N symptoms

silencing signal in grafted plants is not always observed (Dr Peter Waterhouse *pers. comm.*). In order to test whether S+AS and hpRNA transgenes produce a systemic silencing signal capable of mediating PVY resistance in previously susceptible tissues, I set-up a large number of graft combinations. These grafting combinations and the hypotheses they addressed are outlined in Table 3.2.

I adopted reciprocal grafting in my experiments as this was the method used in previous gene silencing studies (Palauqui *et al.* 1997, Palauqui & Vaucheret 1998). Plants used as rootstocks and those used as scions were grown simultaneously. The grafting procedure was performed when the plants reached a height of ~40cm. The spread of silencing from S+AS or hpRNA expressing tissues was tested by PVY challenge and subsequent evaluation of viral symptoms.

The experiments described in Chapter 2 demonstrated that PVY-D derived S+AS and hpRNA transgenes offer very good protection against the 55N strain. In addition, the 55N strain produces easily identifiable symptoms in susceptible tissues (Fig 2.2, A and C, Fig. 3.3). In order to facilitate visual evaluation of viral symptoms I challenged the first set of grafted plants (grafting combinations outlined in Table 3.2) with the 55N strain.

Three weeks post inoculation, the grafts were examined for 55N symptoms (Fig. 2.2 A and C, Fig.3.3). Almost all of the PVY susceptible tissues (WT, S or AS) that were grafted in combination with PVY resistant tissues (S+AS or hpRNA) remained PVY susceptible (results are outlined in Table 3.3, Fig. 3.4). Only tissues expressing the PVY-derived transgene in sense (S) orientation appeared to acquire a degree of

Table 3.2: A summary of questions addressed by reciprocal grafting experiments involving tissues in which expression of S+AS or hpRNA silencing constructs mediates resistance to PVY and tissues that are susceptible to PVY.

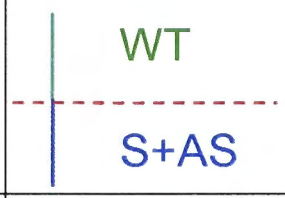
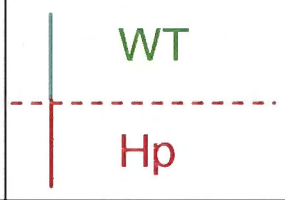
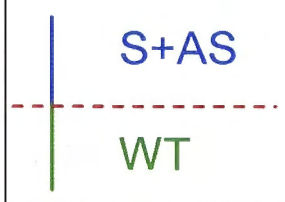
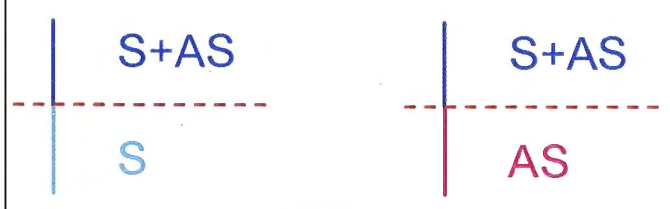
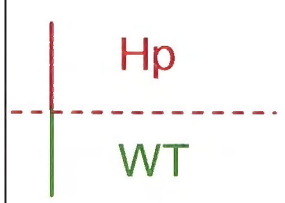
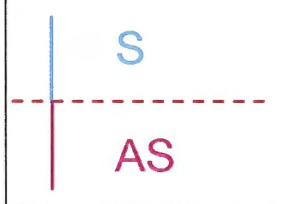
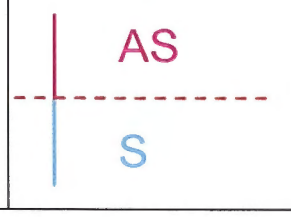
Question	Grafts used to answer the question
Is S+AS rootstock able to induce silencing in WT scion tissues?	
Is S+AS rootstock able to induce silencing in susceptible scion tissues expressing the PVY-derived transgene in either sense (S) or anti-sense (AS) orientation?	
Is hpRNA rootstock able to induce silencing in WT scion tissues?	
Is hpRNA rootstock able to induce silencing in susceptible scion tissues expressing the PVY-derived transgene in either S or AS orientation?	
Is S+AS scion able to induce silencing in WT rootstock tissues?	
Is S+AS scion able to induce silencing in susceptible rootstock tissues expressing the PVY-derived transgene in either S or AS orientation?	
Is Hp scion able to induce silencing in WT rootstock tissues?	
Is Hp scion able to induce silencing in susceptible rootstock tissues expressing the PVY-derived transgene in either S or AS orientation?	
Is S expressing scions able to interact with AS expressing rootstock and induce PVY silencing and consequent resistance?	
Is AS expressing scion are able to interact with S expressing rootstock and induce PVY silencing and consequent resistance?	

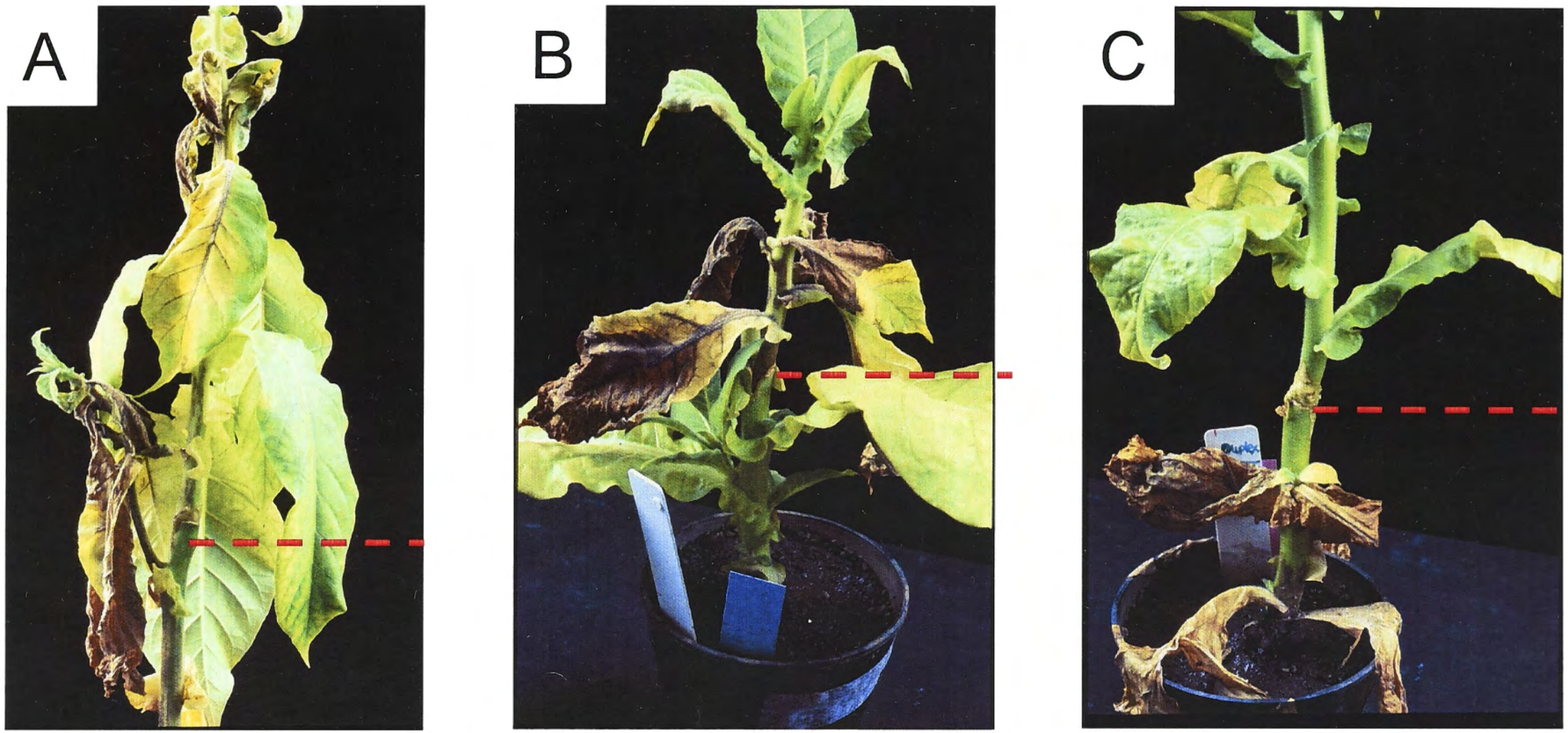
Figure 3.4: Representative examples of reciprocally grafted plants.

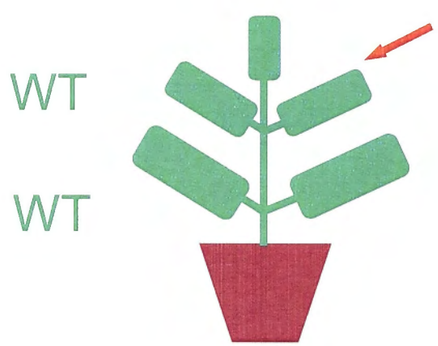
A. WT scion grafted onto WT rootstock.

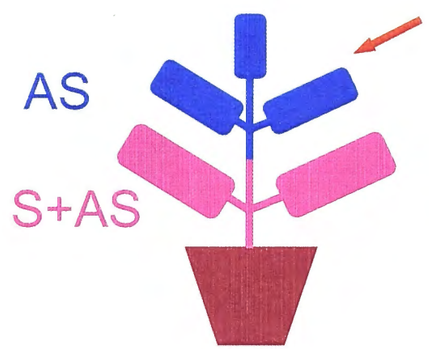
B. AS scion grafted onto S+AS rootstock.

C. S+AS scion grafted onto AS rootstock.

At least 5 grafts were generated for every grafting combination. 3 weeks after the grafting procedure a section of the plant marked with a red arrow was inoculated with the PVY-55N strain. Photographs were taken 4 weeks after the inoculation. The graft junction is marked with a red line.


A
B
C


WT
WT


AS
S+AS

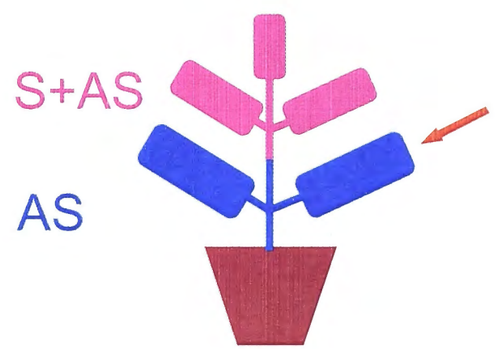

S+AS
AS

Table 3.3: Results from a grafting experiment involving reciprocally grafted plants inoculated with PVY-55N strain.
Site of inoculation for each grafting combination is indicated within the table.

S+AS as a rootstock			
Graft	Inoculation	Infection	Spread of silencing
WT / S+AS	5 x top	5 x infected 5 x no symptoms	NO
S / S+AS	5 x top	1 x few symptoms 4 x infected 5 x no symptoms	NO
AS / S+AS	5 x top	5 x infected 5 x no symptoms	NO

hpRNA as a rootstock			
Graft	Inoculation	Infection	Spread of silencing
WT / Hp	5 x top	5 x infected 5 x no symptoms	NO
S / Hp	5 x top	5 x infected 5 x no symptoms	NO
AS / Hp	5 x top	5 x infected 5 x no symptoms	NO

S or AS as rootstocks			
Graft	Inoculation	Infection	Spread of silencing
S	5 x top	1 x few symptoms 4 x infected	NO
AS		5 x infected	
AS	5 x top	1 x no symptoms 4 x infected	NO
S		3 x infected 2 x no symptoms	

S+AS as a scion			
Graft	Inoculation	Infection	Spread of silencing
S+AS		5 x no symptoms	NO
WT	5 x bottom	5 x infected	
S+AS		5 x no symptoms	NO
S	5 x bottom	5 x infected	
S+AS		5 x no symptoms	NO
AS	5 x bottom	5 x infected	

hpRNA as a scion			
Graft	Inoculation	Infection	Spread of silencing
Hp		5 x no symptoms	NO
WT	5 x bottom	5 x infected	
Hp		5 x none	NO
S	5 x bottom	4 x infected 1 x no symptoms	
Hp		5 x no symptoms	NO
AS	5 x bottom	5 x infected	

S or AS as scions			
Graft	Inoculation	Infection	Spread of silencing
S		1 x few symptoms 4 x infected	NO
AS	5 x bottom	5 x infected	
AS		5 x infected	NO
S	5 x bottom	4 x infected	

CONTROLS			
Graft	Inoculation	Infection	Spread of silencing
WT	5 x top	5 x top and bottom	N/A
WT	5 x bottom	5 x top and bottom	
S	5 x top	5 x top and bottom	N/A
WT	5 x bottom	5 x top and bottom	
WT	5 x top	5 x top and bottom	N/A
S	5 x bottom	5 x top and bottom	
AS	5 x top	5 x top and bottom	N/A
WT	5 x bottom	5 x top and bottom	
WT	5 x top	5 x top and bottom	N/A
AS	5 x bottom	5 x top and bottom	

resistance. However, this was most likely an artifact as the same phenomenon was observed in S tissues that were grafted in combination with wild type tissues. It is possible that the systemic spread of gene silencing was not observed due to the sequence difference between the PVY-D derived transgene from which the signal would be composed and the 55N strain which was inoculated onto the susceptible tissues.

To test this possibility Dr Peter Waterhouse (CSIRO Plant Industry, ACT, Australia) prepared an additional set of grafts that were inoculated with the PVY-D strain. The results from the grafting experiment which involved inoculation with the PVY-D strain were very similar to the results from the grafting experiment in which the plants were inoculated with the 55N strain (Dr Peter Waterhouse *pers. comm.*, Table 3.4). Furthermore, in this experiment, S tissues grafted in combination with either another susceptible tissue, or in combination with a PVY resistant tissue again displayed attenuated viral symptoms. Since symptom attenuation was observed regardless of the graft combination it is most likely due to a degree of auto-protection inherently present in S tissues.

In order to test this possibility, S expressing plants were inoculated with PVY-D. The viral symptoms were observed for 4 weeks after the inoculation. The plants initially displayed PVY symptoms, but at ~2 weeks post inoculation appeared to undergo a recovery (Fig. 3.5). At ~ 4 weeks, viral symptoms were reduced to patches of very mild chlorosis (Fig. 3.5). These observations suggest that S-expressing tissues display a degree of auto-protection and therefore are not suitable for the evaluation of the systemic spread of silencing in further grafting experiments.

Table 3.4: Results from a grafting experiment involving reciprocally grafted plants inoculated with PVY-D strain.
Site of inoculation for each grafting combination is indicated within the table.

S+AS as a rootstock			
Graft	Inoculation	Infection	Spread of silencing
WT / S+AS	5 x top	5 x infected 5 x no symptoms	NO
S / S+AS	5 x top	4 x infected 1 x no symptoms 5 x no symptoms	NO
AS / S+AS	5 x top	5 x infected 5 x no symptoms	NO

S+AS as a scion			
Graft	Inoculation	Infection	Spread of silencing
S+AS / WT	1 x bottom	1 x no symptoms 1 x infected	NO
S+AS / S	5 x bottom	5 x no symptoms 5 x infected	NO
S+AS / AS	5 x bottom	5 x no symptoms 5 x infected	NO

S or AS as rootstocks			
Graft	Inoculation	Infection	Spread of silencing
S	5 x top	3 x no symptoms 2 x infected	NO
AS		3 x infected 2 x no symptoms	
AS	5 x top	5 x infected	NO
S		4 x infected 1 x no symptoms	

hpRNA as a rootstock			
Graft	Inoculation	Infection	Spread of silencing
WT	5 x top	5 x infected	NO
Hp		5 x no symptoms	
S	5 x top	5 x infected	NO
Hp		5 x no symptoms	
AS	5 x top	5 x infected	NO
Hp		5 x no symptoms	

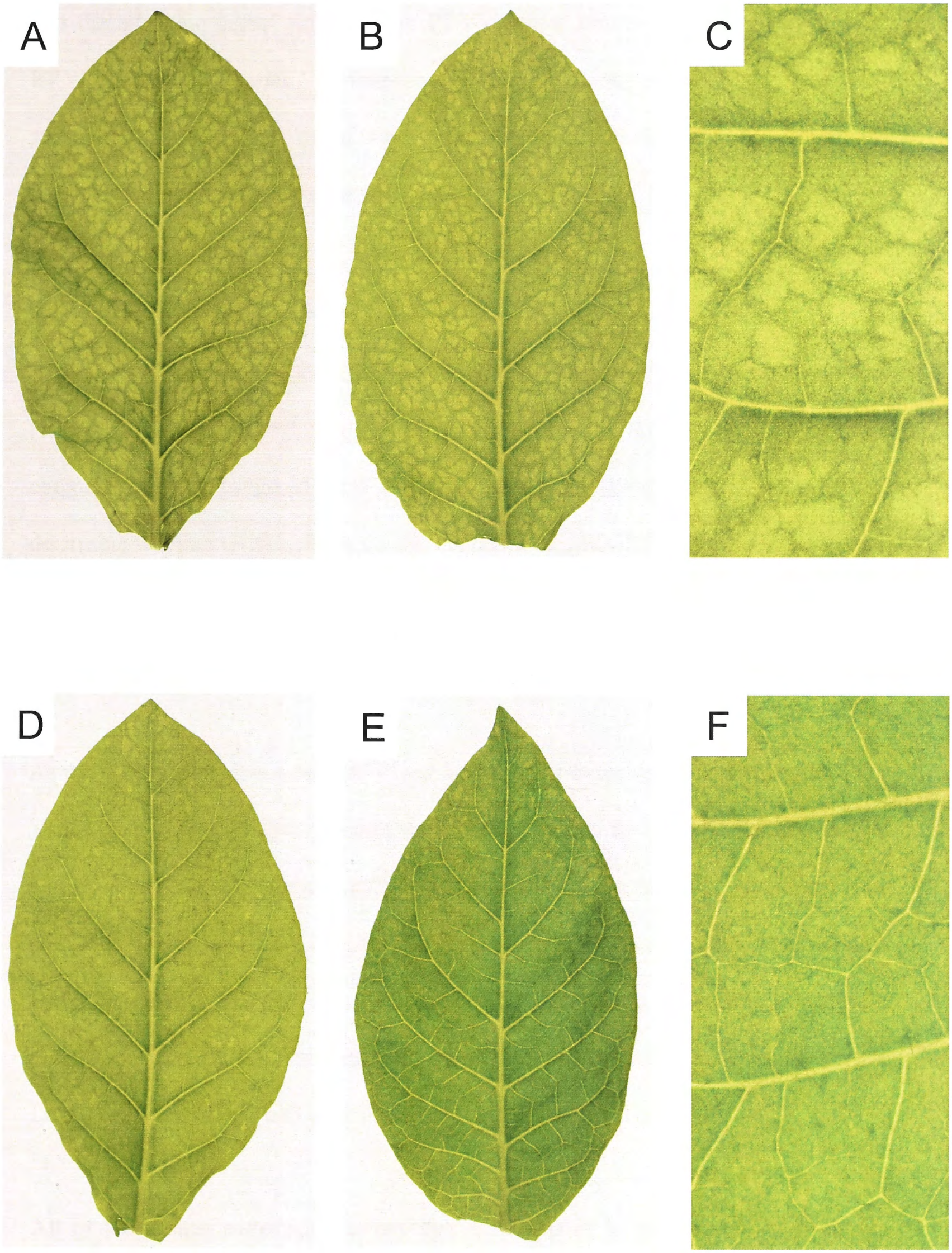
hpRNA as a scion			
Graft	Inoculation	Infection	Spread of silencing
Hp		5 x no symptoms	NO
WT	5 x bottom	5 x infected	
Hp		5 x no symptoms	NO
S	5 x bottom	4 x infected 1 x no symptoms	
Hp		5 x no symptoms	NO
AS	5 x bottom	5 x infected	

S or AS as inducers of silencing			
Graft	Inoculation	Infection	Spread of silencing
S	2 x bottom	2 x no symptoms	NO
AS		2 x infected	
AS	3 x bottom	3 x infected	NO
S		2 x infected 1 x no symptoms	

CONTROLS			
Graft	Inoculation	Infection	Spread of silencing
S	5 x top	1 x infected 4 x no symptoms	N/A
WT		3 x infected	
WT	5 x bottom	4 x infected	N/A
S		5 x infected	
AS	5 x top	5 x infected	N/A
WT		4 x infected 1 x no symptoms	
WT	5 x top	5 x infected	N/A
AS	5 x bottom	5 x infected	

Figure 3.5: Recovery phenotype observed in plants expressing the PVY-derived transgene in the sense orientation (S plants).

Eight small (2-3 leaves) S plants were inoculated with PVY-D. Leaves shown in panels A and B are typical of symptoms developed by these plants two weeks post inoculation. Panel C shows widespread vein clearing observed in these leaves. At four weeks post inoculation S plants showed a marked reduction in symptoms. Leaves shown in panels D and E show typical symptoms for this time. No obvious vein clearing was detected in such leaves (panel F).


A
B
C
D
E
F

Taken together, the observations from the two sets of grafting experiments suggest that the silencing signal generated by PVY-resistant tissues did not spread into the PVY susceptible tissues. Alternatively, the silencing signal could reach the susceptible tissues, but could not induce a response required for onset and maintenance of silencing in such tissues.

3.3.2 Systemic spread of GUS silencing in reciprocal grafts

Systemic spread of GUS silencing has previously been reported by Palauqui *et al.* (1997). However, the GUS-silenced plant lines used in these experiments carried complex loci that triggered GUS suppression in early development. We aimed to determine whether GUS hpRNA constructs could trigger GUS silencing when grafted in combination with GUS expressing tissues. The grafting combinations and the questions they addressed are outlined in Table 3.5.

As the GUS protein has a relatively long half-life (Dr Peter Waterhouse and Dr Vicky Vance *pers. comm.*), I allowed eight weeks before testing for GUS silencing in tissues that expressed GUS before the grafting procedure. Leaf-disc samples were taken from one of the leaves below the grafting point and the four leaves above the grafting point. Only the youngest leaf below the grafting point was tested as leaves further down the plant were entering into the senescent phase and showed variable GUS expression. Leaf discs were stained and evaluated for GUS expression (Table 3.6).

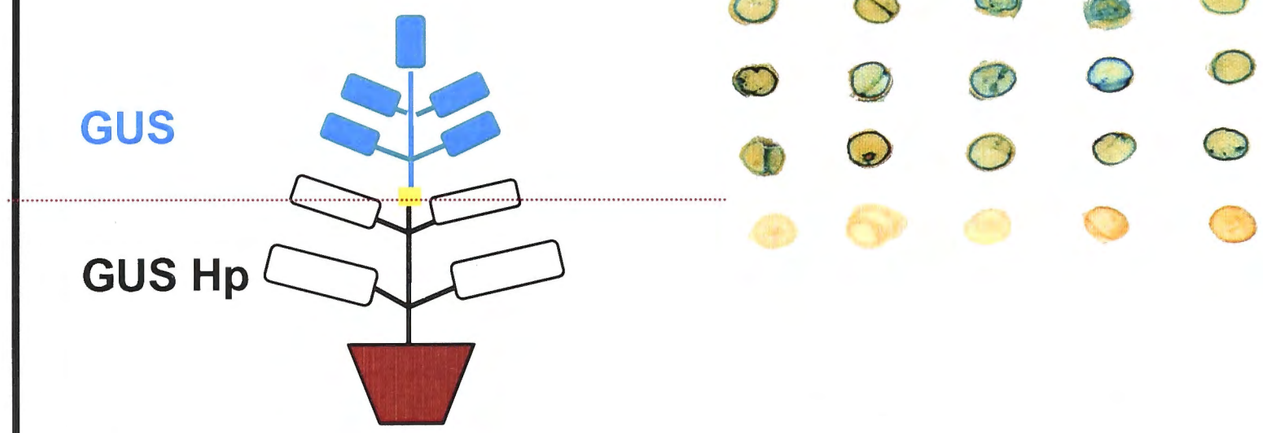
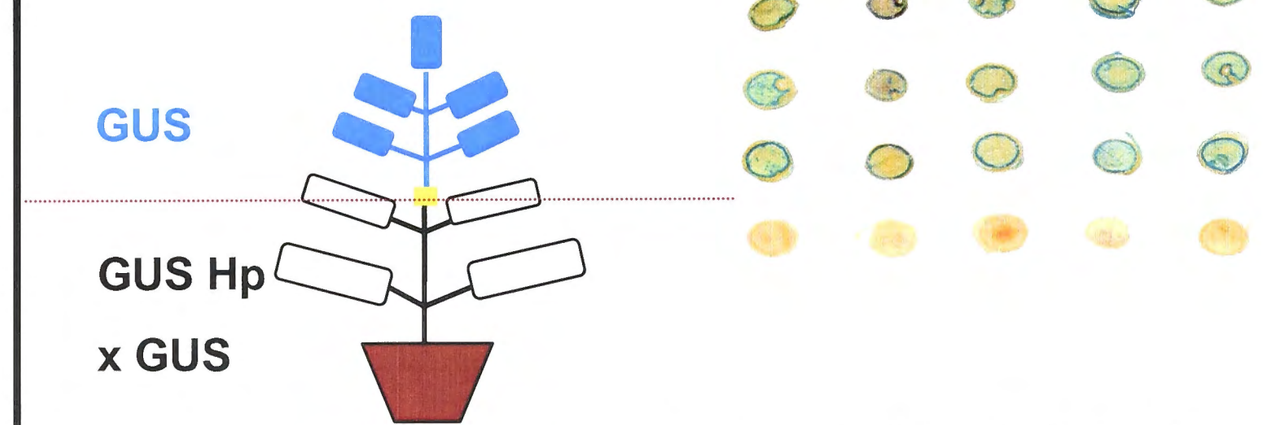
All of the tissues with GUS activity that were grafted in combination with tissues expressing the GUS hairpin RNA (GUS Hp), or the GUS anti-sense construct, retained

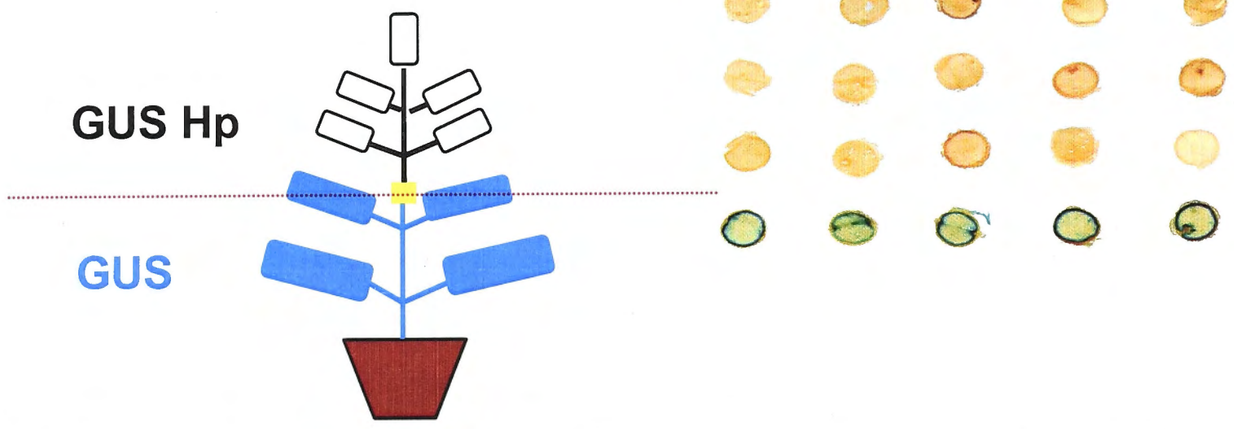
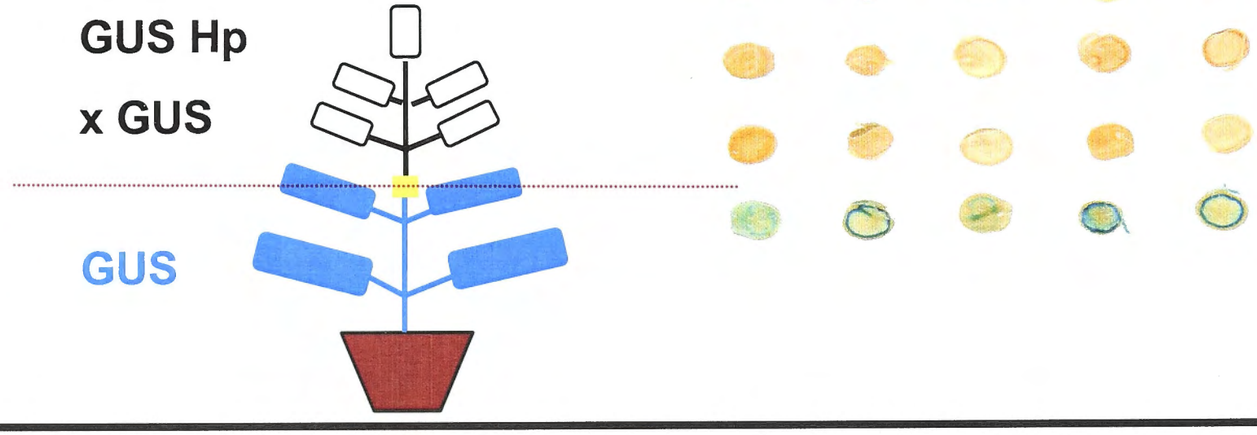
Table 3.5: A summary of questions addressed by reciprocal grafting experiments involving tissues expressing a GUS silencing construct and tissues expressing an active GUS protein.

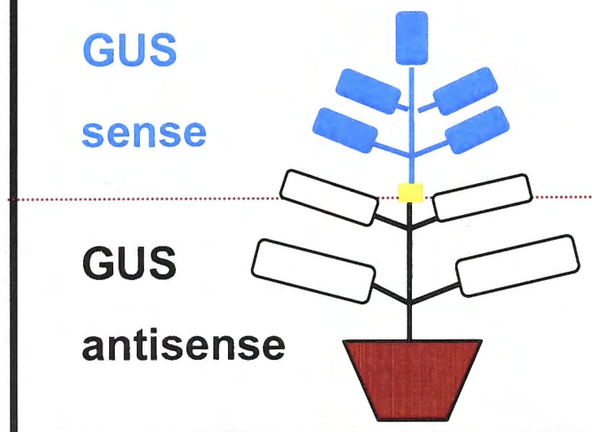
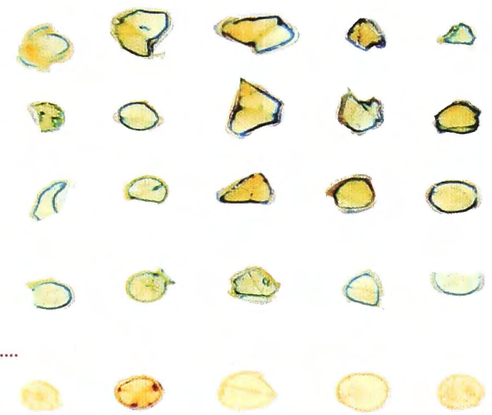
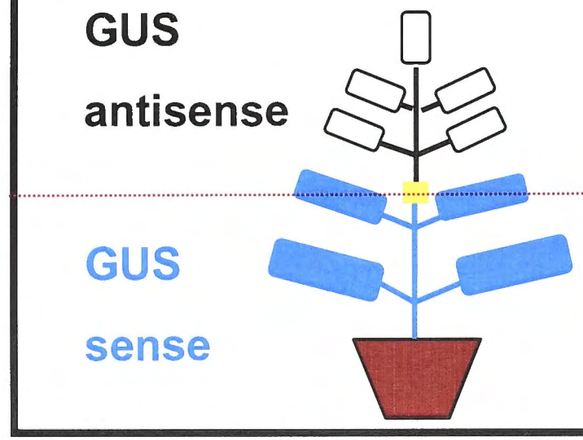
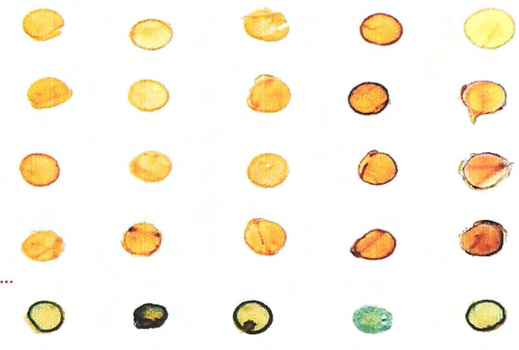
Question	Grafts used answer the question
Is GUS Hp rootstock able to induce silencing in GUS expressing scion tissues?	GUS GUS Hp
Is GUS-silenced rootstock tissue transcribing both GUS RNA and GUS Hp RNA able to induce silencing in GUS expressing scion tissues? Is a silenced tissue containing both silencing construct (GUS Hp) and its target (GUS) a more efficient inducer of systemic silencing?	GUS GUS x GUS Hp
Is GUS anti-sense (AS) expressing rootstock able to interact with GUS expressing scion and induce GUS silencing in the scion?	GUS GUS AS
Is GUS Hp scion able to induce silencing in GUS expressing rootstock tissues?	GUS Hp GUS
Is GUS-silenced scion tissue transcribing both GUS RNA and GUS Hp RNA able to induce silencing in GUS expressing scion tissues? Is a silenced tissue containing both silencing construct (GUS Hp) and its target (GUS) a more efficient inducer of systemic silencing?	GUS x GUS Hp GUS
Is GUS anti-sense (AS) expressing scion able to interact with GUS expressing rootstock and induce GUS silencing in the rootstock?	GUS AS GUS

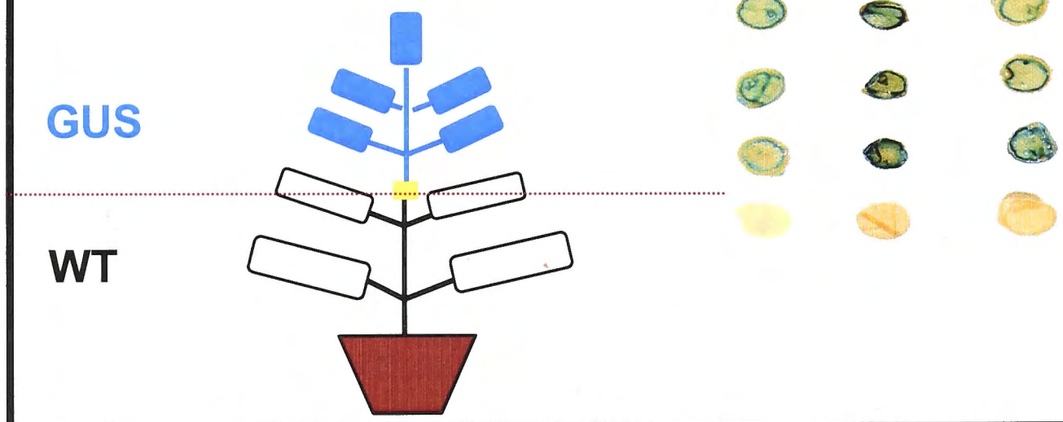
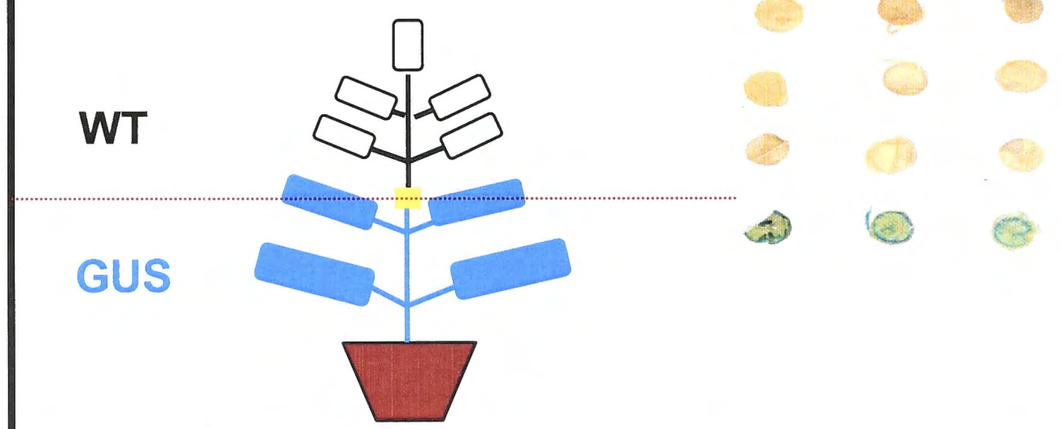
Table 3.6: Results from a reciprocal grafting experiment involving tissues expressing a GUS silencing construct and tissues expressing an active GUS protein.

Five grafted plants were used to test each experimental question (see Table 3.5). For each grafted plant, leaf disc samples were taken from one of the leaves below the graft junction and the four leaves above the graft junction. Leaf discs were stained for GUS expression as described in section 2.2.5.

Graft	Result
	No spread
	No spread

Graft	Result
	No spread
	No spread

Graft	Result
GUS sense  GUS antisense 	No spread
GUS antisense  GUS sense 	No spread

Graft	Result
GUS WT 	N/A (control)
WT GUS 	N/A (control)

GUS activity. GUS silenced tissues, expressing both GUS Hp construct and its GUS RNA target, were also unable to induce silencing in tissues with GUS activity.

Therefore, using a reciprocal grafting method, we were not able to detect any systemic spread of silencing of viral genes (PVY) or transgenes (GUS). This suggests that S+AS and hairpin RNA constructs may not be able to produce a silencing signal capable of eliciting a silencing response in fully developed tissues expressing the target RNA.

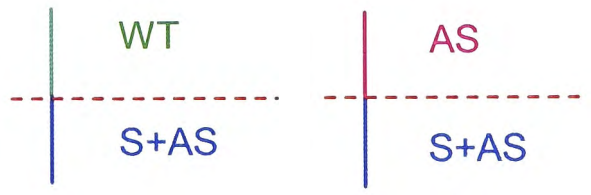
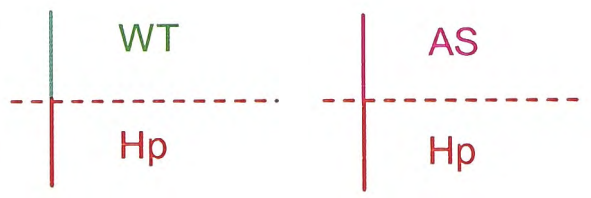
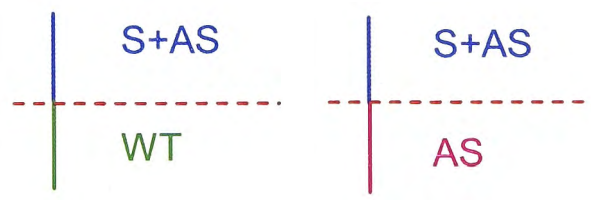
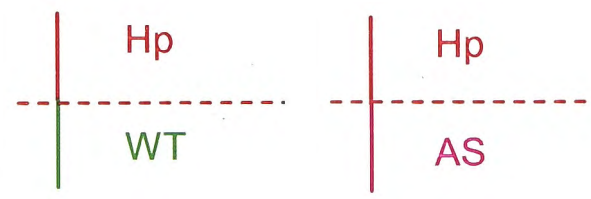
3.3.3 Systemic spread of PVY silencing in top grafts

After the completion of our reciprocal grafting experiments Crete *et al.* (2001) published a study suggesting that graft transmission of PTGS was dependent on the grafting technique. The authors stated that graft transmissible silencing could not be detected in plants that were grafted using reciprocal or plug grafting methods. In contrast, efficient spread of silencing was observed in plants that were grafted using the top grafting method.

In the light of findings described by Crete *et al.* (2001) I hypothesised that our inability to detect graft transmissible silencing in previous experiments could have been due to the grafting technique. Therefore, the experiments described in Section 3.3.1 were repeated using the top grafting method.

Two sets of top grafts (grafting combinations outlined in Table 3.7) were prepared. One set was inoculated with the PVY-D strain, while the other was inoculated with 55N strain. PVY infection was detected and quantified by ELISA analysis. These

Table 3.7: A summary of questions addressed by top grafting experiments involving tissues resistant to PVY and tissues susceptible to PVY.

Question	Grafts used answer the question
Is S+AS rootstock able to induce silencing in WT or AS-expressing scion tissues?	
Is hpRNA rootstock able to induce silencing in WT or AS-expressing scion tissues?	
Is S+AS scion able to induce silencing in WT or AS-expressing rootstock tissues?	
Is Hp scion able to induce silencing in WT or AS-expressing rootstock tissues?	

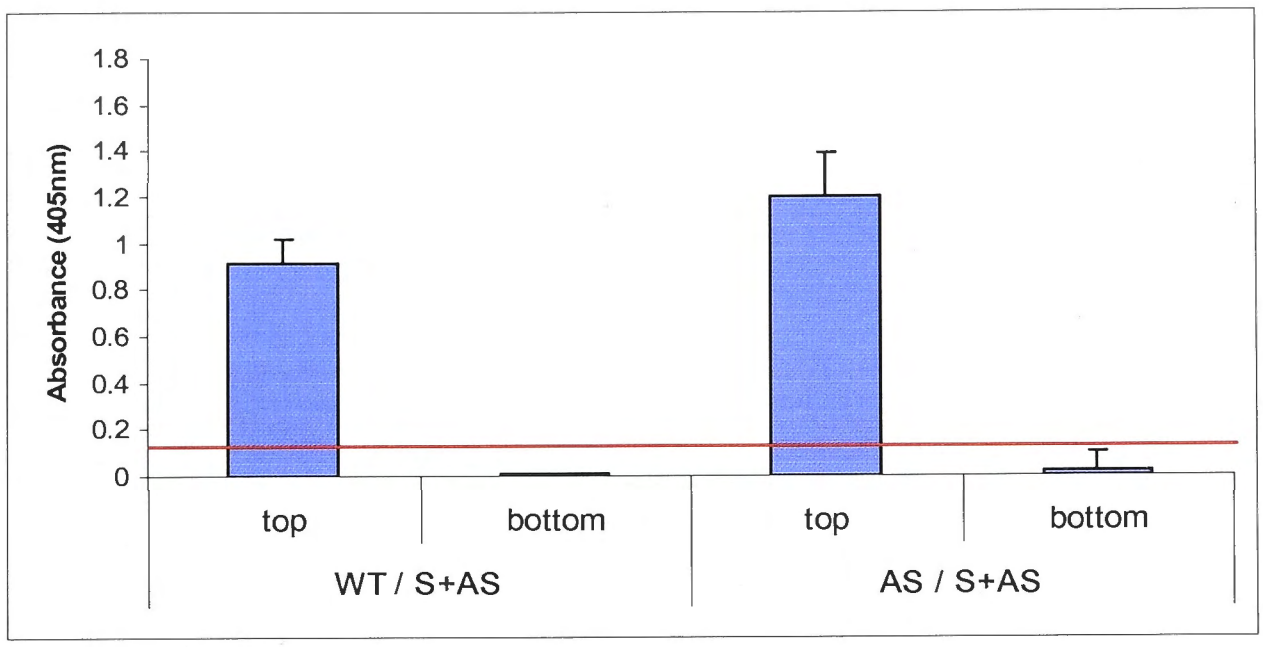
data were used to evaluate graft transmissible silencing from S+AS or hpRNA expressing tissues into unprotected tissues.

S+AS-expressing rootstocks did not induce a significant degree of protection in PVY-D inoculated WT or AS-expressing scions (Fig. 3.6, A). The virus levels in WT and AS scions grafted onto S+AS rootstocks were comparable to viral levels detected in WT and AS scions grafted onto a WT rootstock (Fig. 3.6, A,C). However, AS-expressing scions grafted onto hpRNA-expressing rootstock displayed a high degree of resistance to PVY-D (Fig. 3.6, B). WT scions grafted onto hpRNA rootstocks did not develop complete resistance to PVY-D, but accumulated less virus then the control grafts (WT scion grafted onto WT rootstock) (Fig. 3.6, B,C).

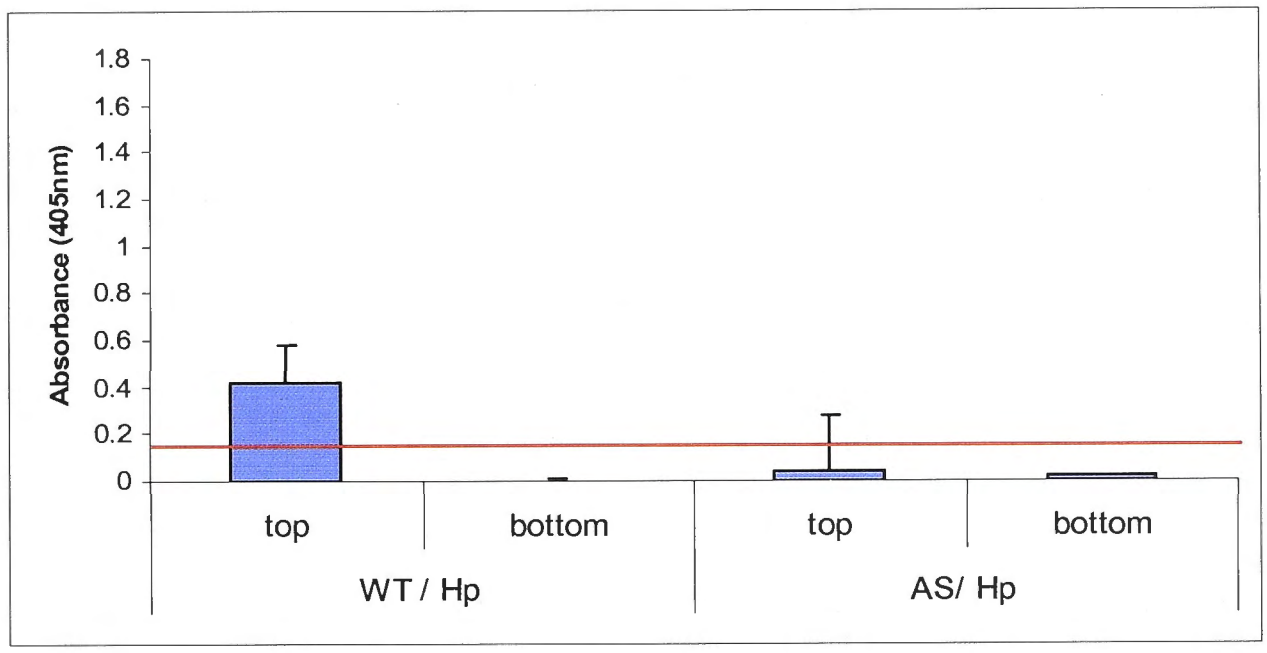
However, neither S+AS nor hpRNA rootstocks were able to induce resistance to the 55N strain in WT or AS-expressing scions (Fig. 3.7). This finding was surprising because previous experiments established that: 1) the hpRNA transgene could generate a mobile silencing signal capable of triggering PVY silencing in grafted tissues; and 2) the hpRNA transgene was able to provide very good protection against the 55N strain when the virus was inoculated onto a tissue expressing the transgene.

S+AS and hpRNA scions were not able to mediate protection against either of the PVY strains. All WT and AS rootstocks grafted in combination with S+AS or hpRNA scions accumulated the virus to levels comparable to those detected in control grafts (WT rootstocks grafted onto WT scions) (Fig. 3.8, Fig. 3.9).

A



B



C

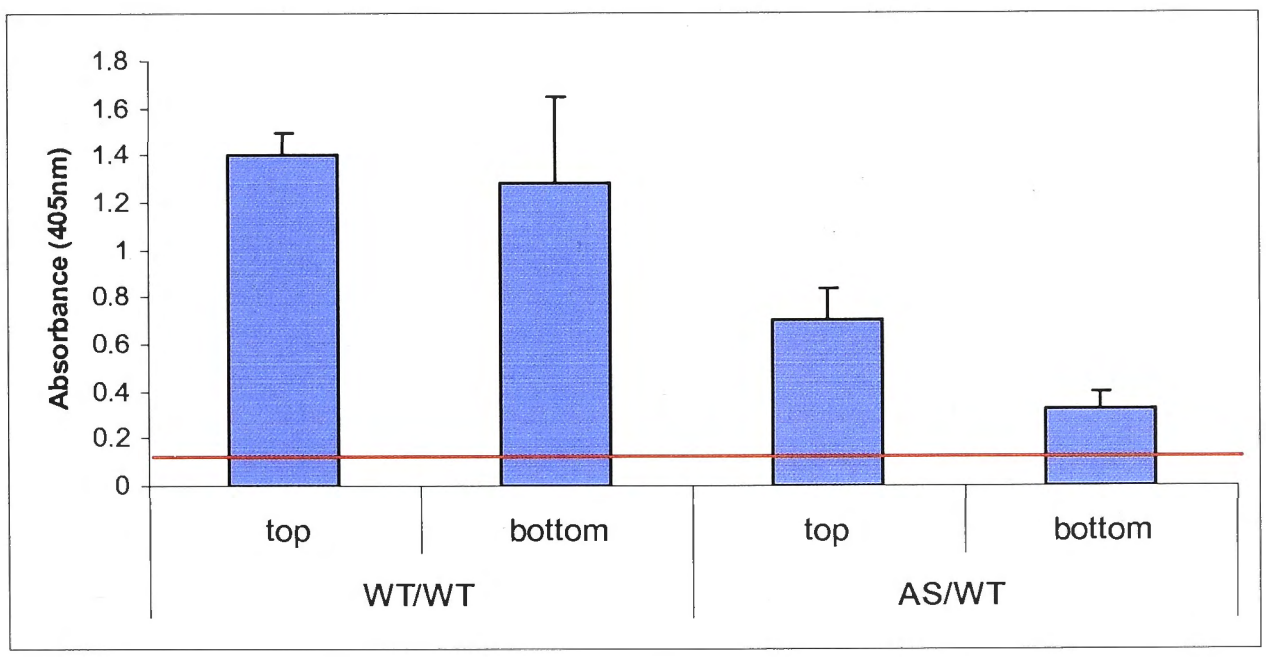


Figure 3.6: Inoculation of the scion portion of top grafted plants with PVY-D.

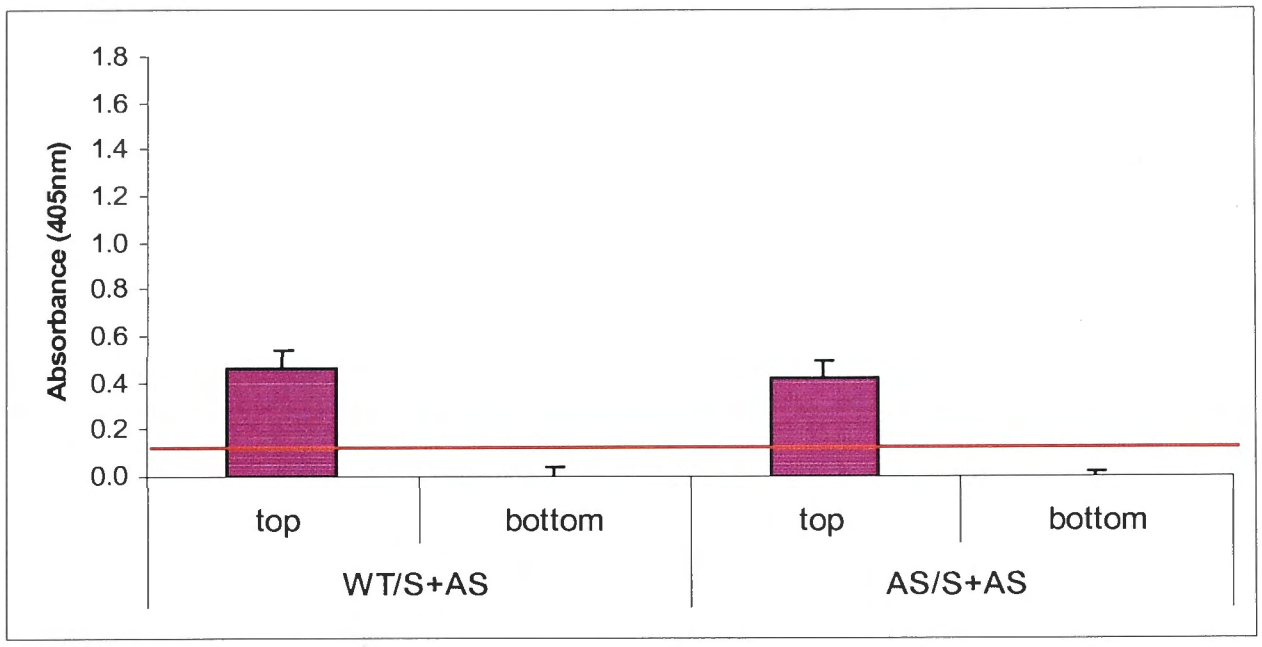
A. WT and AS scions top grafted onto S+AS rootstocks.

B. WT and AS scions grafted onto Hp rootstocks.

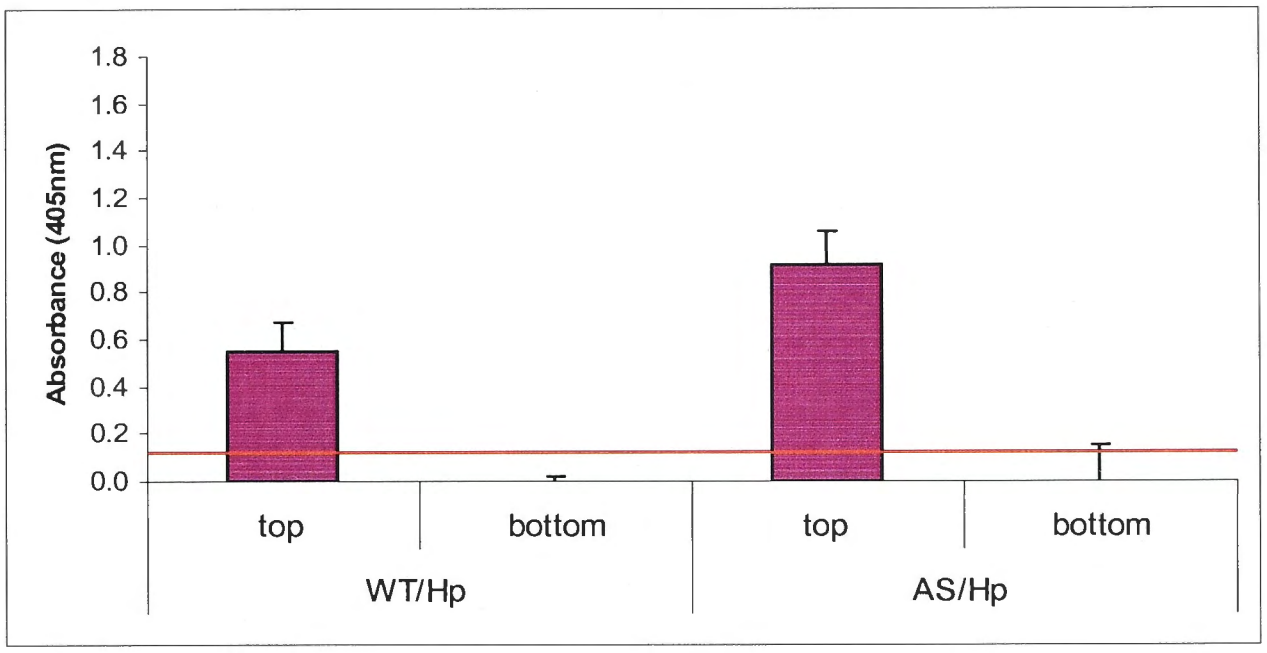
C. WT and AS scions grafted onto WT rootstocks were used as experimental control.

At least 10 grafts were generated for every grafting combination. Grafts were inoculated with PVY-D strain 3 weeks after grafting and analysed by ELISA 3 weeks after the inoculation. Red line represents A405nm reading above which plants are considered to be infected. The columns represent median values while error bars are derived from standard error values.

A



B



C

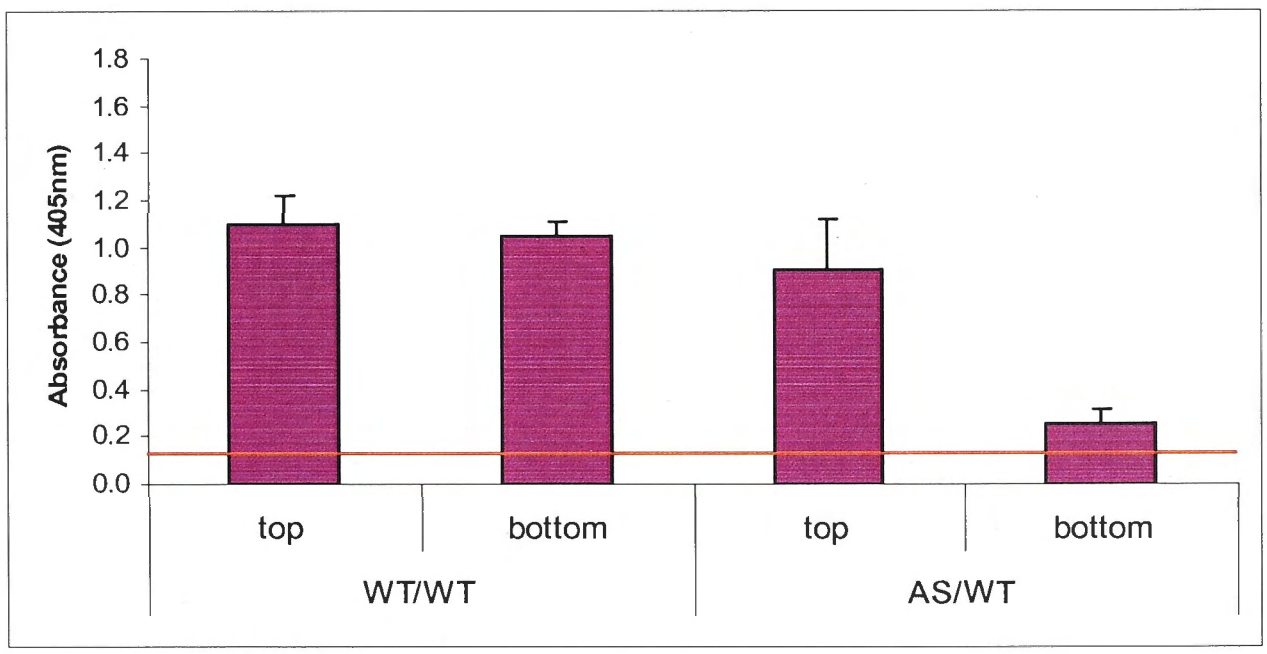


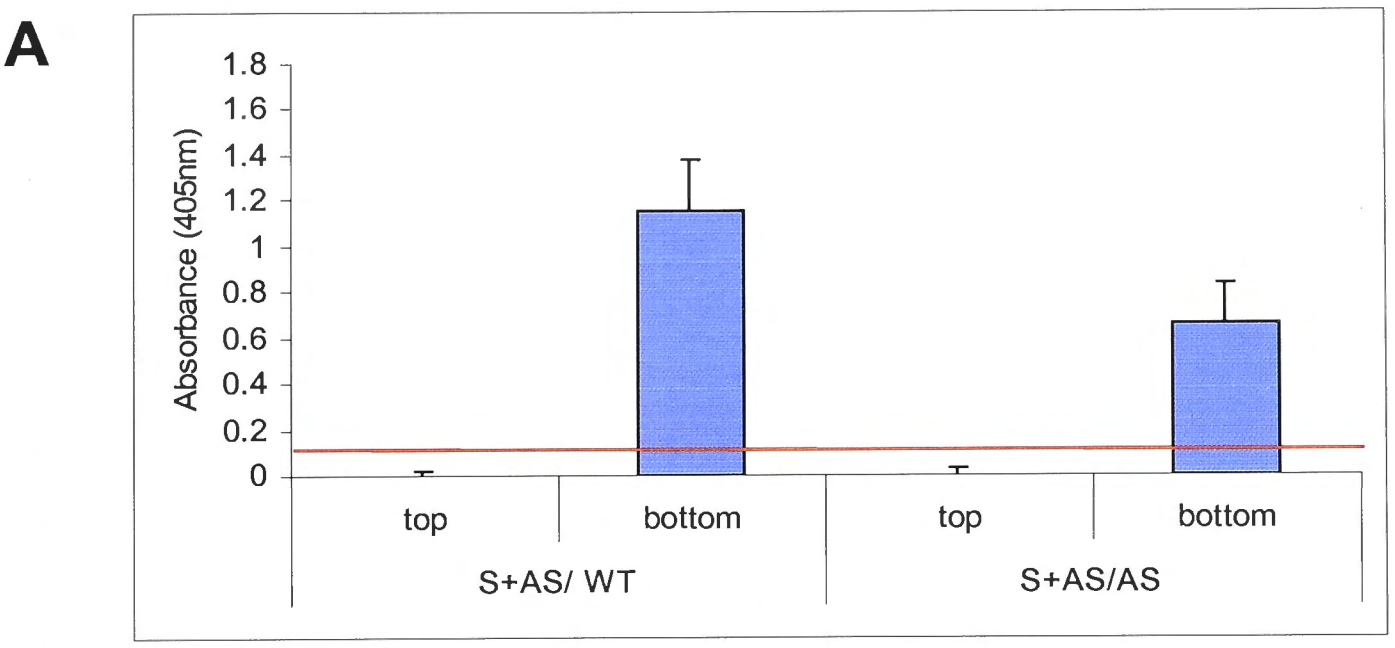
Figure 3.7: Inoculation of the scion portion of top grafted plants with PVY-55N.

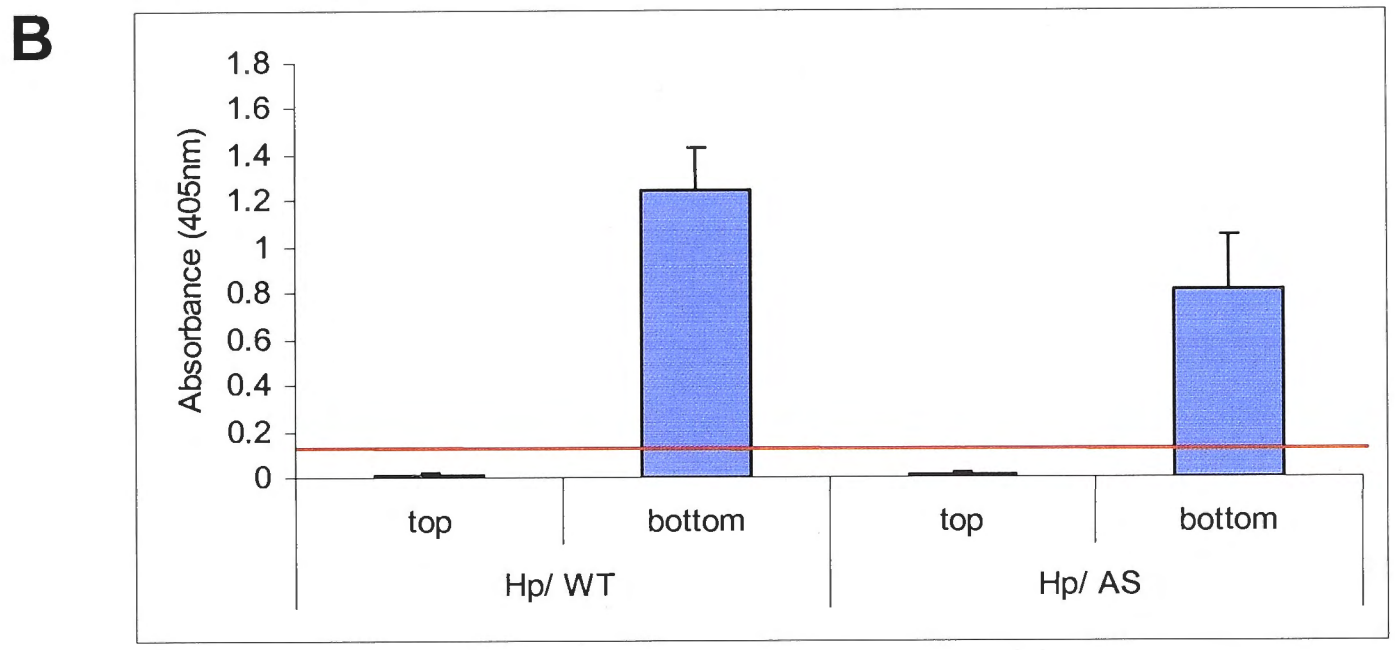
A. WT and AS scions top grafted onto S+AS rootstocks.

B. WT and AS scions grafted onto Hp rootstocks.

C. WT and AS scions grafted onto WT rootstocks were used as experimental control.

At least 10 grafts were generated for every grafting combination. Grafts were inoculated with PVY-55N strain 3 weeks after grafting and analysed by ELISA 3 weeks after the inoculation. Red line represents A405nm reading above which plants are considered to be infected. The columns represent median values while error bars are derived from standard error values.





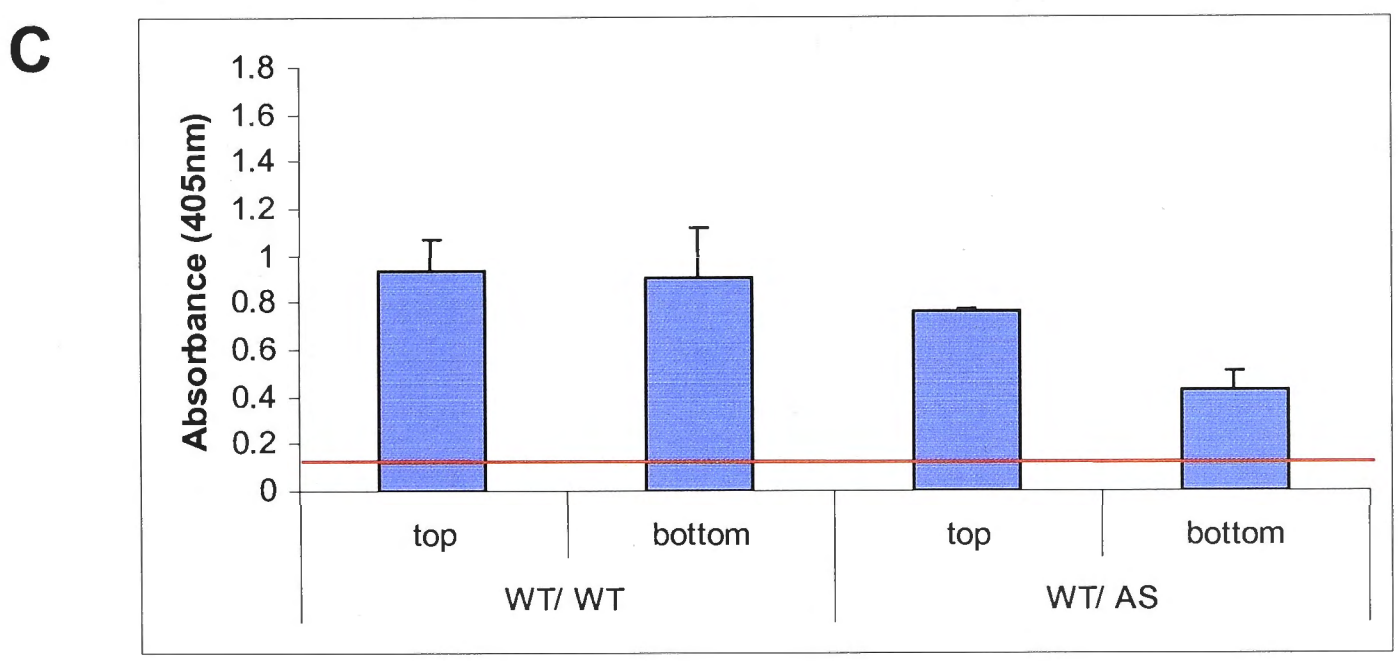


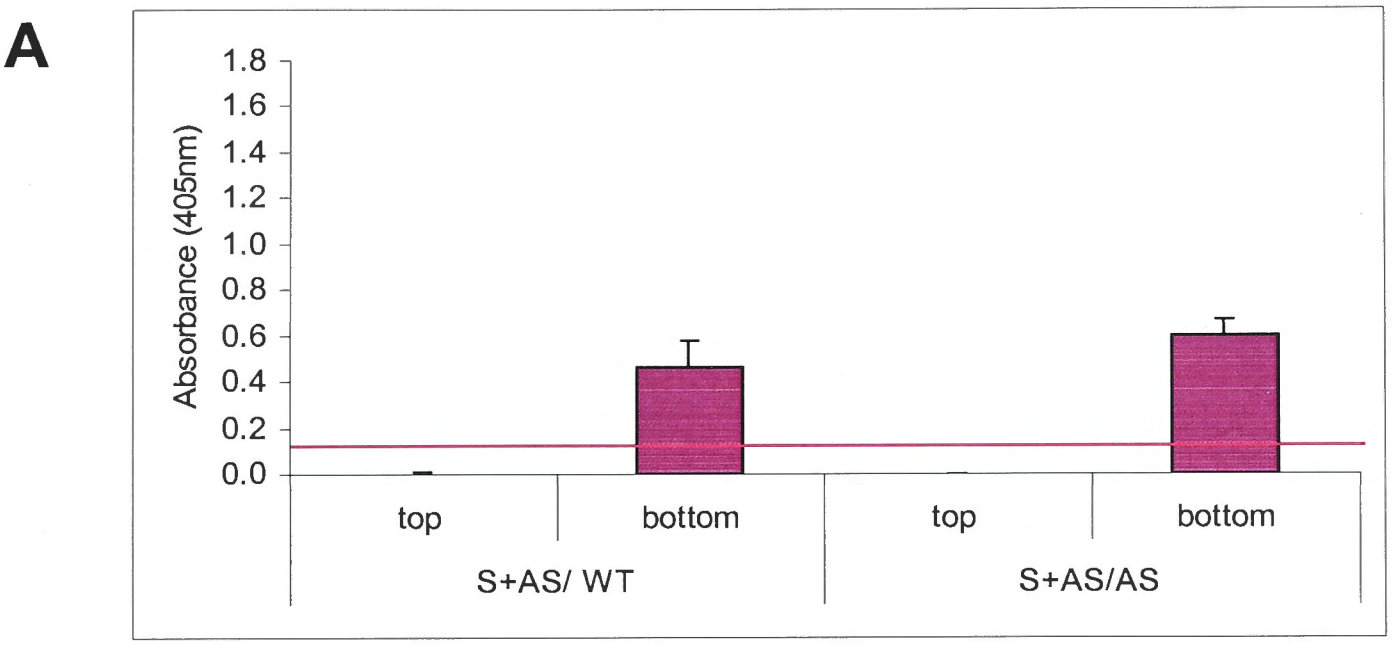
Figure 3.8: Inoculation of the rootstock portion of top grafted plants with PVY-D.

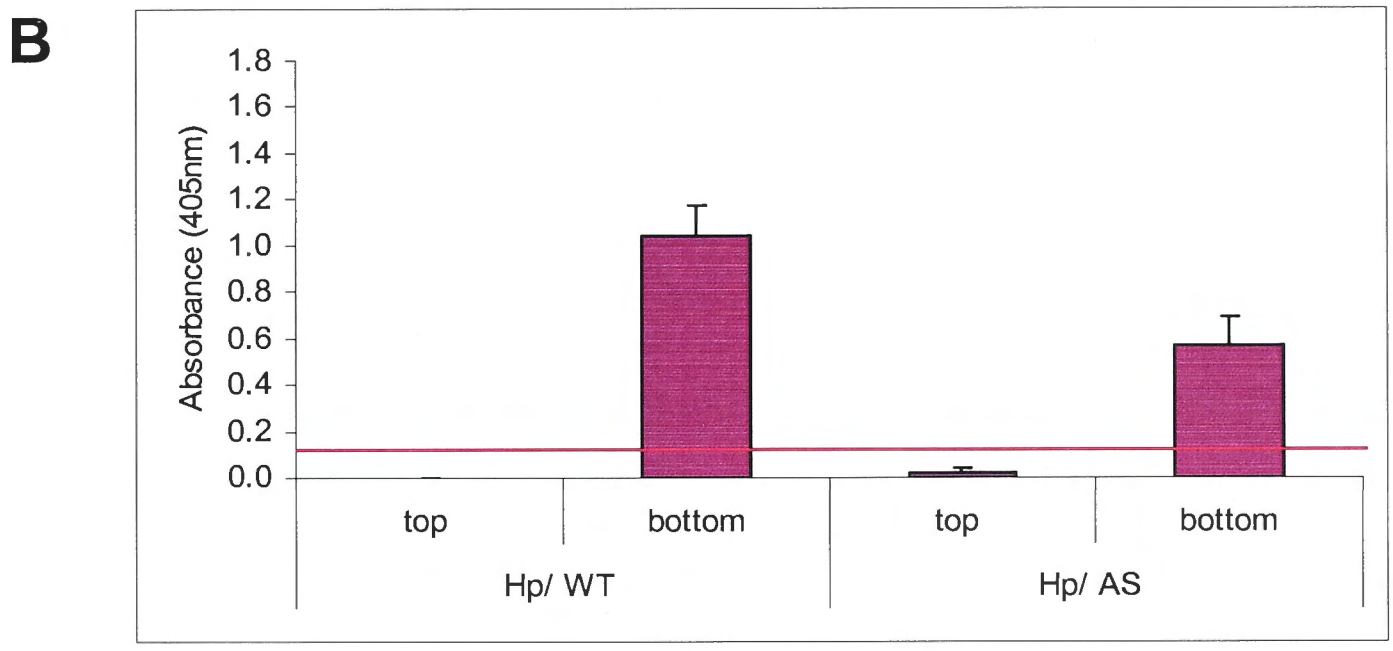
A. S+AS scions top grafted onto WT and AS rootstocks.

B. Hp scions top grafted onto WT and AS rootstocks.

C. WT scions grafted onto WT and AS rootstocks were used as experimental control.

At least 10 grafts were generated for every grafting combination. Rootstock sections of grafted plants were inoculated with PVY-D strain 3 weeks after grafting and analysed by ELISA 3 weeks after the inoculation. Red line represents A405nm reading above which plants are considered to be infected. The columns represent median values while error bars are derived from standard error values.





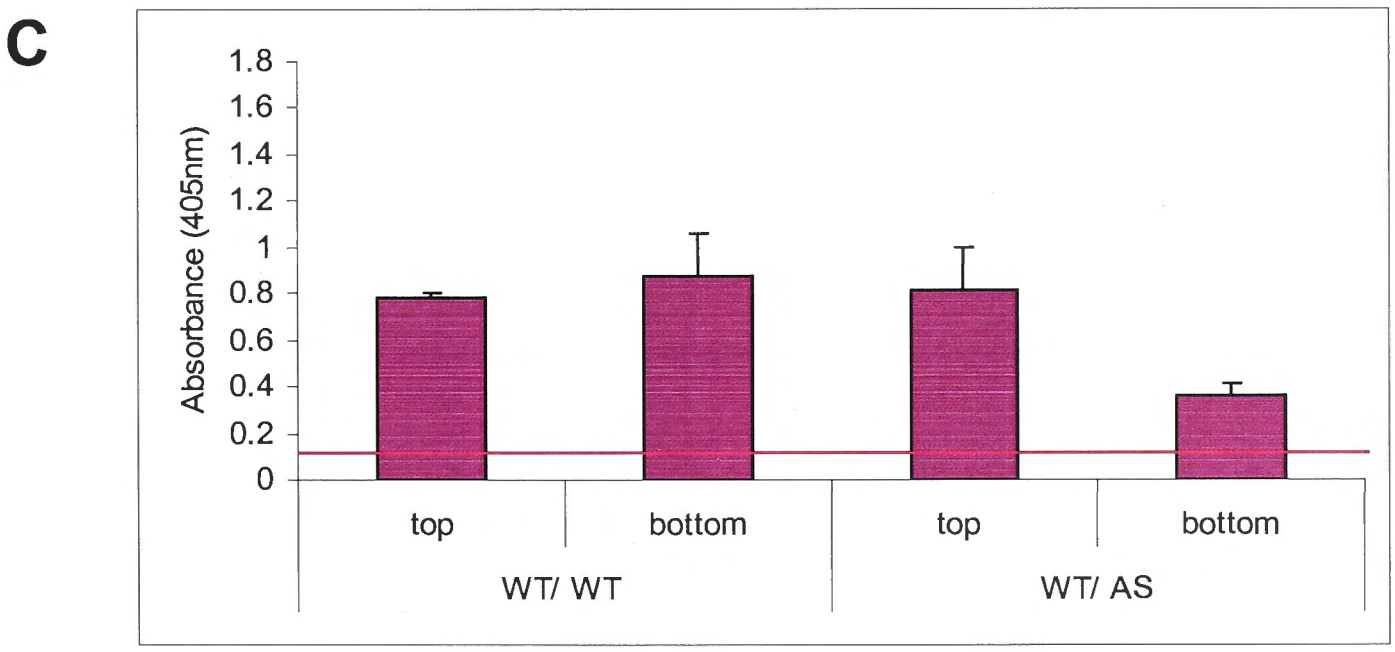


Figure 3.9: Inoculation of the rootstock portion of top grafted plants with PVY-55N.

A. S+AS scions top grafted onto WT and AS rootstocks.

B. Hp scions top grafted onto WT and AS rootstocks.

C. WT scions grafted onto WT and AS rootstocks were used as experimental control.

At least 10 grafts were generated for every grafting combination. Rootstock sections of grafted plants were inoculated with PVY-55N strain 3 weeks after grafting and analysed by ELISA 3 weeks after the inoculation. Red line represents A405nm reading above which plants are considered to be infected. The columns represent median values while error bars are derived from standard error values.

These observations indicate that hpRNA-expressing tissues are able to generate a graft transmissible silencing signal. However, the ability of this signal to mediate PTGS was dependent on the tissue that received the signal. AS-expressing young scion tissue was able to respond to the signal and develop resistance to PVY-D while WT tissues and older tissues seem to be unable to do so.

3.3.4 Systemic spread of GUS silencing in top grafts

In order to test whether the silencing signal produced by a tissue expressing hpRNA could induce effective silencing of other transgenes, I top-grafted GUS-silenced tissues in combination with GUS expressing tissues (Table 3.8). As in previous experiments, I did not detect any spread of silencing from silenced scions into expressing rootstocks, we focused on investigation of signal transmission from rootstocks to scions.

After discussions with Dr Hervé Vaucheret, I altered the method for analysis of GUS expression in the scion tissues. Eight weeks after grafting the three uppermost scion leaves were harvested. To ensure complete penetration of GUS stain during the staining procedure small leaves were prepared by cutting off the leaf edges while the larger leaves were cut into 2 to 3 sections. The tissues were vacuum infiltrated with the GUS stain, incubated in the same stain overnight and de-stained as described in section 2.2.5.

GUS silencing was detected in the vascular tissue of GUS-expressing scions grafted onto GUS Hp rootstocks (Fig. 3.10). In older leaves, silencing was limited to major veins, while in the very young leaves it had also spread through the minor veins. A

Table 3.8: A summary of questions addressed by top grafting experiments involving GUS-silenced and GUS expressing tissues.

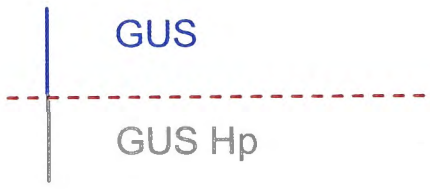
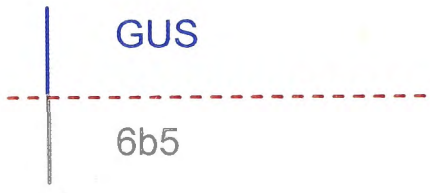
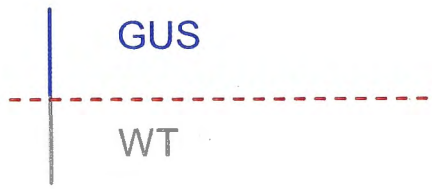
Question	Grafts used answer the question
Is GUS Hp rootstock able to induce silencing in GUS expressing scion tissues?	
Is GUS-silenced rootstock tissue transcribing both GUS RNA and GUS Hp RNA able to induce silencing in GUS expressing scion tissues? Is a silenced tissue containing both silencing construct (GUS Hp) and its target (GUS) a more efficient inducer of systemic silencing?	
CONTROL	
CONTROL	

Figure 3.10: Representative examples of GUS silencing observed in top grafted plants.

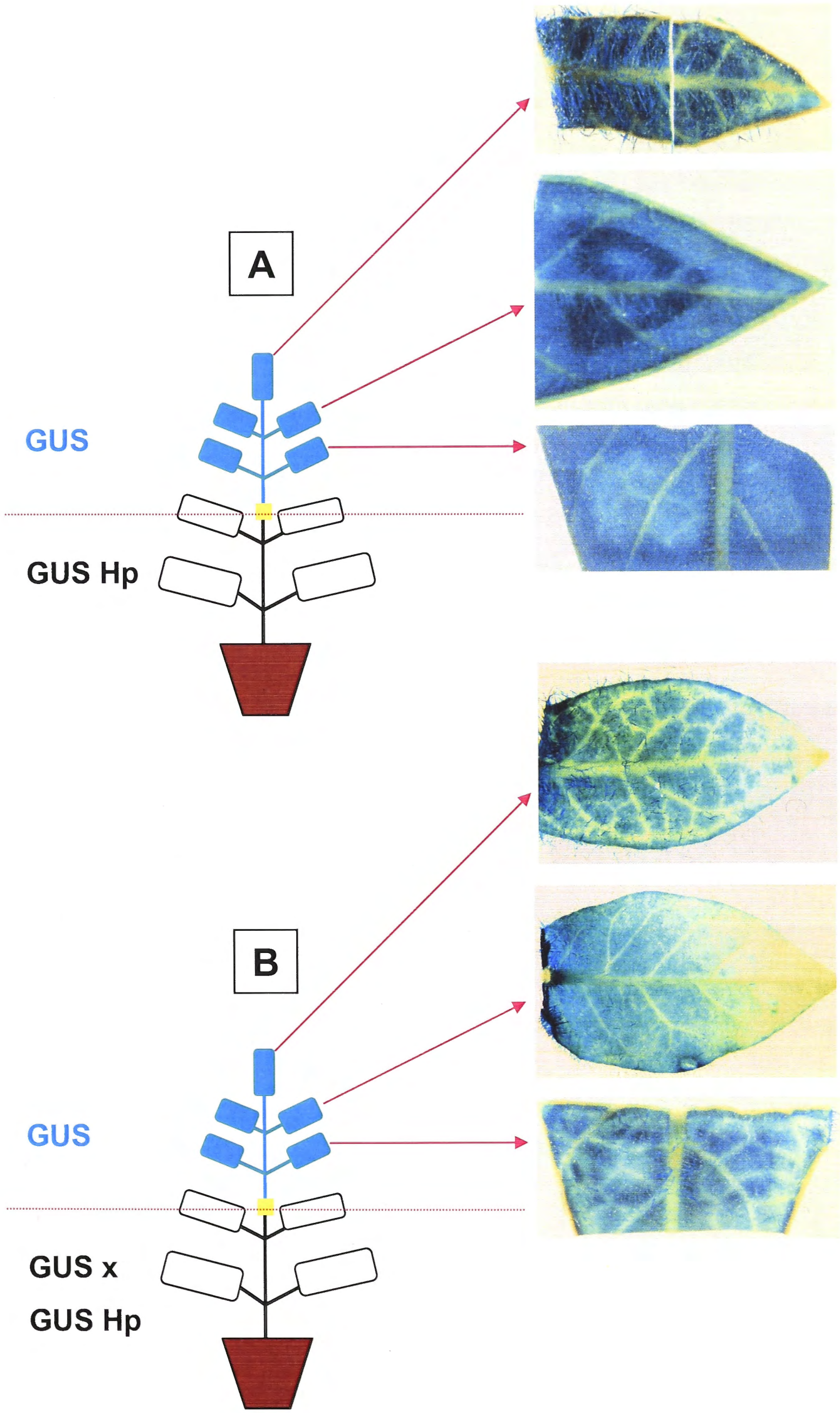
A. Scion expressing GUS grafted onto a rootstock expressing the GUS Hp construct.

B. Scion expressing GUS grafted onto a rootstock in which the GUS gene had already been silenced by the GUS Hp construct.

C. Scion expressing GUS grafted onto 6b5 rootstock in which GUS gene has been silenced by co-suppression (positive control).

D. Scion expressing GUS grafted onto WT rootstock (negative control).

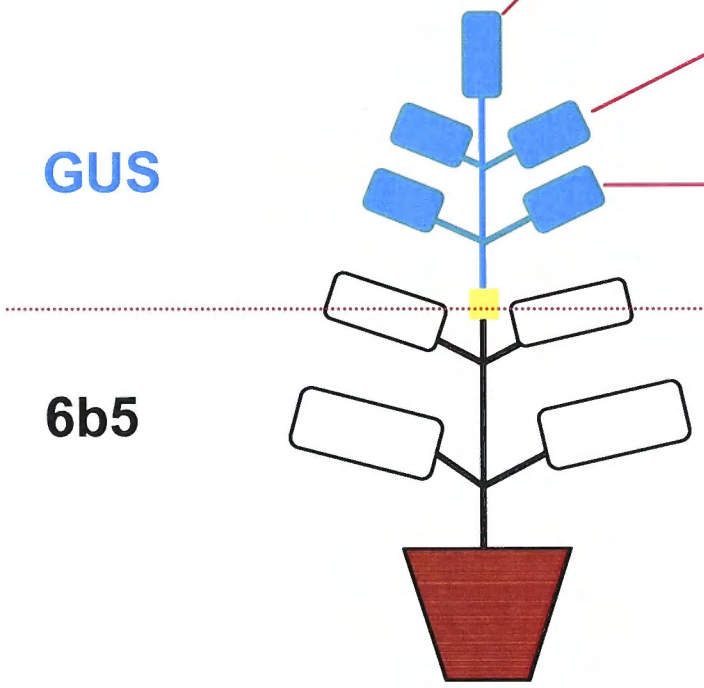
At least 10 grafts were generated for every grafting combination. Eight weeks after the grafting procedure the top three leaves were removed from the scion. The leaves were vacuum infiltrated with GUS stain, incubated at 37°C overnight and de-stained in 70% ethanol. The dotted red line represents a graft junction while red arrows mark the leaves that were removed from the plant and stained.

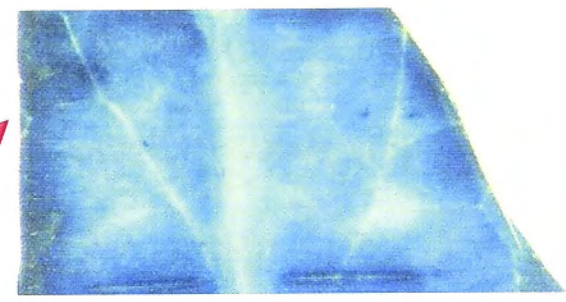

A
GUS
GUS Hp
B
GUS
GUS x
GUS Hp

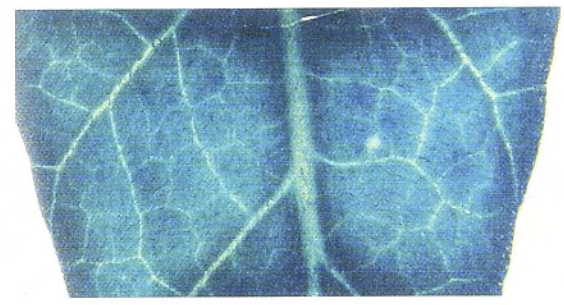
C

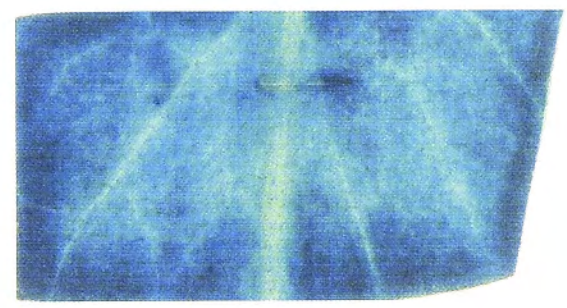
GUS

6b5





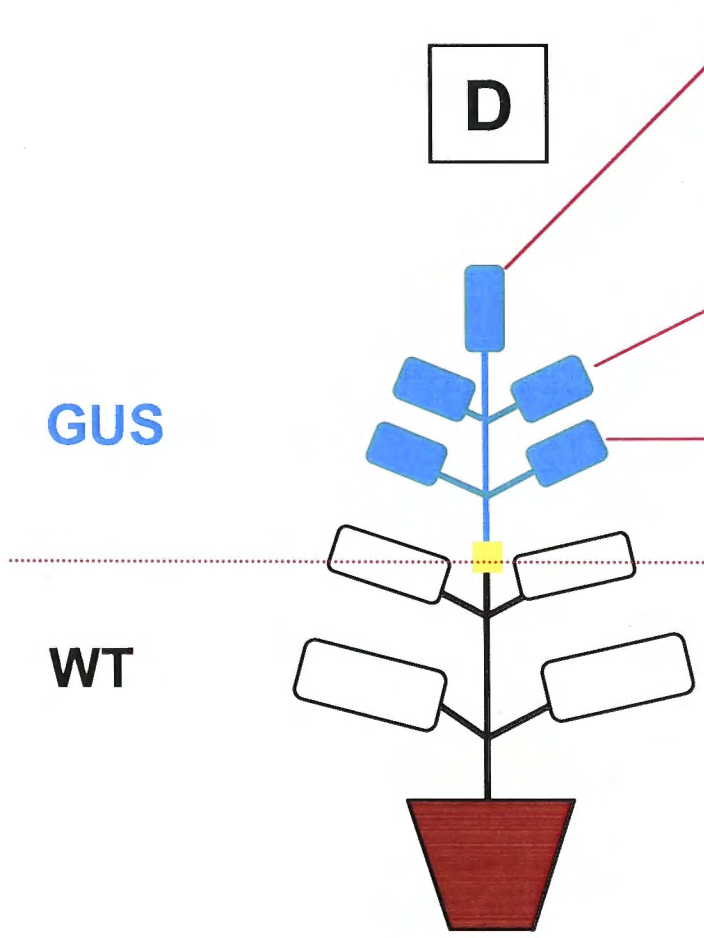


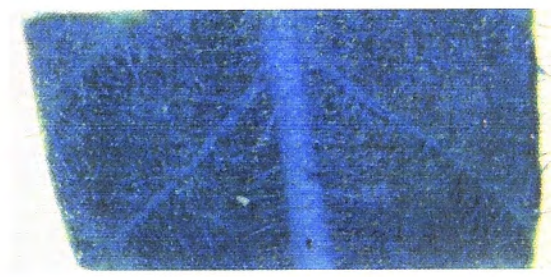


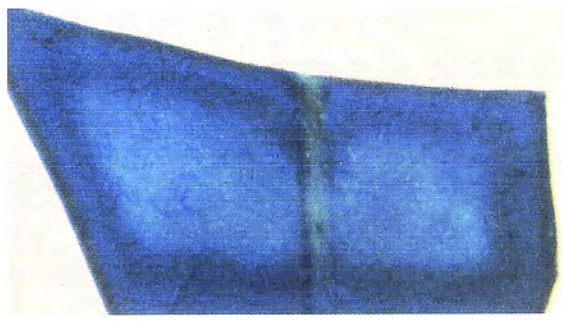
D

GUS

WT







similar silencing pattern was observed in scions grafted onto the 6b5 plant line. More pronounced GUS silencing was observed in scions grafted onto rootstocks that were transcribing both the GUS Hp construct and GUS mRNA. In some such grafts GUS silencing also spread out of the vasculature and into the mesophyll tissue (Fig. 3.10). The control grafts (GUS expressing scions grafted onto WT rootstocks) did not display any GUS silencing, indicating that the grafting procedure itself did not have an effect on GUS expression.

These observations indicate that a GUS Hp construct can produce a graft transmissible silencing signal which can induce a silencing response in GUS expressing tissues. Interestingly, the presence of the GUS target within the GUS Hp expressing tissue appears to increase the potency of the silencing signal.

3.4 Discussion

The experiments described in this chapter show that PTGS can spread from the silenced tissues into the tissues expressing the corresponding transgene. The spread of PTGS was affected by the grafting method, occurred only in one direction (from the rootstock into the scion) and was dependent on the structure of the inducing transgenes and the extent of homology between the inducing transgene and its target (Fig. 3.11).

The efficiency of systemic spread of PTGS was highly dependent on the grafting technique. Systemic spread of PVY and GUS silencing was observed in top-grafted plants, but not in plants grafted using the reciprocal grafting technique. This finding is consistent with a report showing that chitinase silencing could be transmitted by top grafting but not by reciprocal or plug grafting (Crete *et al.* 2001).

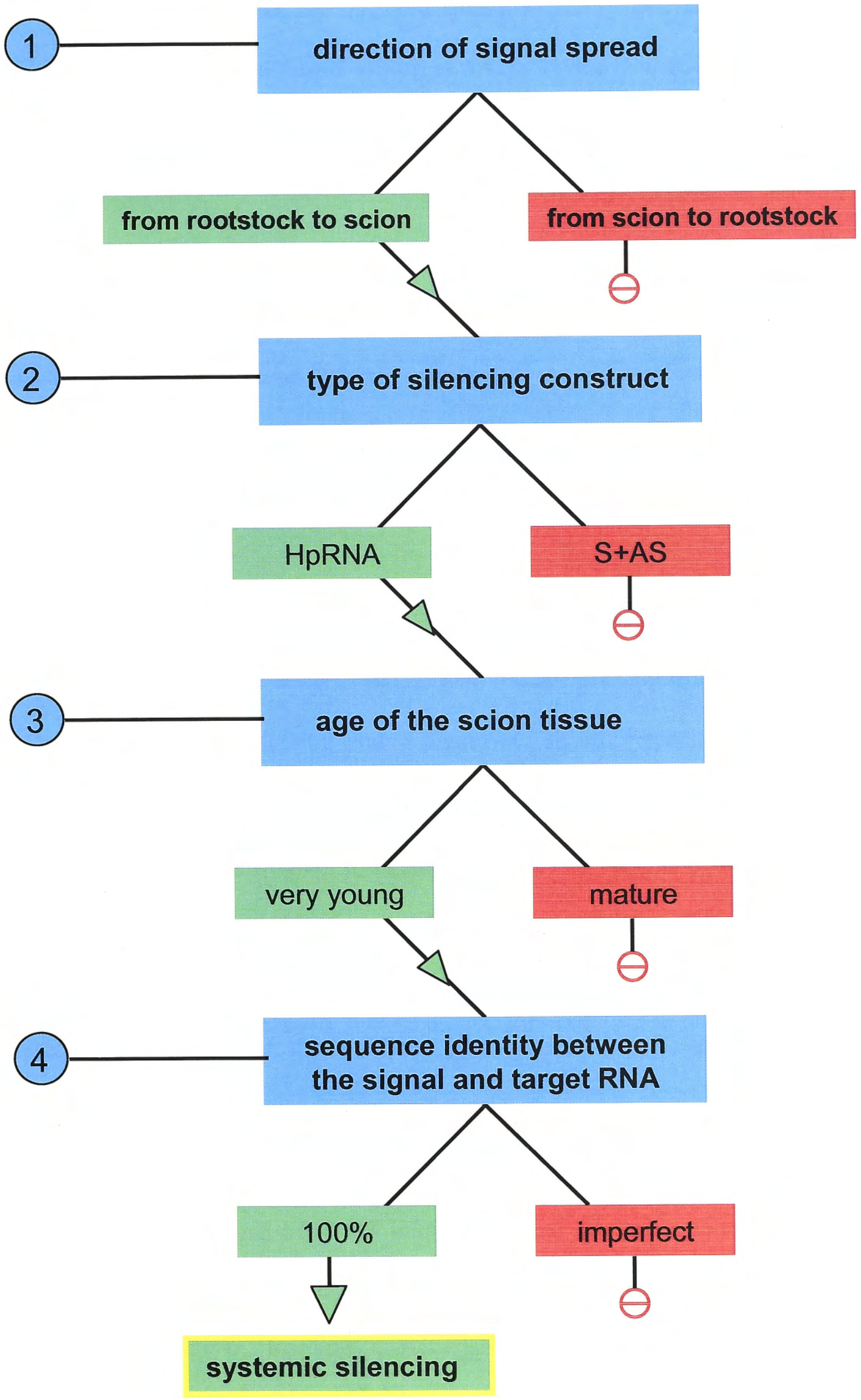
Figure 3.11: Factors influencing the systemic spread of PTGS in grafted plants.

1. Direction of signal spread.
   The spread of PTGS occurred only in one direction - from the rootstock into the scion.

2. Type of silencing construct.
   hpRNA transgenes produced a transmissible silencing signal while S+AS transgenes were not able to do so.

3. Age of the scion tissue.
   Age of the scion tissue depended on the grafting method. The spread of PTGS was only observed when very young scion tissue was top-grafted onto the silenced rootstocks.

4. Sequence identity between the signal and the target RNA.
   The spread of PTGS was only observed when there was 100% sequence identity between the transgene from which the signal was derived and the target RNA.


1
direction of signal spread
from rootstock to scion
from scion to rootstock
2
type of silencing construct
HpRNA
S+AS
3
age of the scion tissue
very young
mature
4
sequence identity between the signal and target RNA
100%
imperfect
systemic silencing

Crete *et al.* (2001) proposed that the efficient spread of PTGS into the top-grafted scions is due to the young scion tissues acting as a very strong sink for the silencing signal that is produced by the mature leaves of the rootstock and distributed to the rest of the plant via phloem. However, even when the reciprocal or plug grafting methods are used, strong sink tissues are present, although at a greater distance from the source leaves. Previous studies have shown that the silencing signal can be distributed throughout the plant, and can travel through a 30cm long stem. Therefore, the distance from the source is not likely to be solely responsible for the ineffective spread of silencing in reciprocally or plug-grafted plants (Palauqui *et al.* 1997).

It is important to note that both the reciprocal grafting and the exchange of tissue plugs involve mature tissues acting as scions. In contrast, the top grafting involves transfer of upper leaves and meristem from a young plant onto an older rootstock in which silencing is already established. As such, the age of the scion may also play an important role in determining whether systemic silencing can be established. Perhaps the tissues need to be very young to be able to respond to the silencing signal. Evidence supporting this hypothesis comes from biolistic experiments in which it was demonstrated that silencing of the chitinase gene could only be established in seedlings that had less than 7 leaves (Crete *et al.* 2001).

The observation that the spread of PTGS is unidirectional is consistent with several previous studies (Palauqui *et al.* 1997, Palauqui & Vaucheret 1998, Crete *et al.* 2001). The possible explanations for this observation are very similar to those that could account for the effect of the grafting technique - one relates to the amount of signal

received by the target tissue, while the other relates to the ability of the tissue to respond to the signal. Firstly, if the silencing signal, like the green fluorescent protein (GFP), dyes and viruses, moves through phloem, from mature source leaves into the developing sink leaves, the mature leaves of the rootstock are less likely to become exposed to the silencing signal. In addition, even if the mature tissues become exposed to an adequate amount of the silencing signal, they may not be able to respond and use the signal to establish silencing.

The systemic transport of the green fluorescent protein (GFP) is well characterised and resembles the pattern of systemic spread of PTGS. For example, the initial pattern of nitrate reductase silencing in sink tobacco leaves appears in the vicinity of class III veins (Boerjan *et al.* 1994, Palauqui *et al.* 1996). Similarly, GFP synthesised in the companion cells of a source leaf is transported to sink leaves and where it exits phloem from the same vein class suggesting that trafficking of GFP and the silencing signal could be mechanistically related (Oparka *et al.* 1999). As such, the experiments describing the movement of GFP may help us understand analyse the patterns of spread of the silencing signal.

Studies of GFP trafficking indicate that plasmodesmata of mature leaves are less permissive to untargeted protein transport. In leaves undergoing sink-to-source transition GFP (27kD) can pass through only ~20% of the cells while in sink leaves it can be transported through ~70% of cells (Crawford & Zambryski 2001). Larger proteins, for example GFP dimer (54kD), can access only ~2% of the cells in the sink leaves. This difference is probably due to the structural and physiological characteristics of plasmodesmata. In young sink leaves, most plasmodesmata have a

simple, one channel structure, tend to be open and allow for the untargeted passage of large protein complexes. In contrast, in source leaves most plasmodesmata have a more complex, branched structure, which restricts protein trafficking (Oparka *et al.* 1999).

These observations suggest that the amount of the silencing signal, composed of RNA in complex with proteins, reaching mature leaves could be limited, not only by the direction of the phloem flow, but also by leaf physiology. However, it is still not clear whether mature tissues could establish silencing if they had access to an adequate amount of the silencing signal. It would be interesting to investigate whether silencing can spread to strong sink tissues of the rootstock, such as roots or leaves covered with paper bags.

Observations that PTGS does not spread into the developing scion leaves of reciprocally or plug-grafted plants suggest that the presence of the strong sink tissue and the ability of this tissue to receive the signal are not sufficient for the establishment of silencing. Perhaps there is an internal clock that prevents all tissues, young and mature, from an older plant from acquiring silencing. However, the observation that PTGS is more efficient in mature plants than in the young plantlets (see Section 2.3.4), appears to contradict this hypothesis. It is tempting to speculate that there could be a developmental stage during which silencing is being established but is not yet efficient. Once the plant progresses beyond this developmental stage, effective silencing cannot be established. This proposal could be challenged by the observation that mature non-transgenic plants can establish PTGS in response to a viral infection, albeit too late to prevent the infection. However, as suggested by the

experiments described in Section 2.3.7, viral infection could up-regulate silencing responses in plants and enable plants to establish PTGS regardless of the developmental stage.

The systemic spread of PTGS into top-grafted scions was also dependent on the structure of the transgene that mediated silencing in the silenced rootstock tissue. It appears that the hpRNA transgene, but not the S+AS transgenes, were able to produce and send the systemic silencing signal. Perhaps this could be explained by the fact that tissues expressing the hpRNA transgene accumulate a large amount of siRNAs, which are likely to act as a silencing signal, prior to the inoculation with a virus (Fig. 2.1). In contrast, tissues expressing S+AS transgenes produce only a small amount of siRNAs prior to viral inoculation. It is only after viral inoculation that S+AS plants accumulate larger amounts of siRNAs. Consequently, S+AS rootstocks, which have not been challenged with a virus, may not have a sufficient amount of siRNAs and therefore may not be able to send them in sufficient concentration to trigger silencing in the unprotected tissues.

When a plant composed of S+AS rootstock and WT or AS scion is challenged with a virus, the scions become infected, but the infection does not spread into the S+AS rootstock. It is likely that PTGS becomes up-regulated as S+AS tissues encounter the virus. When this happens, a large amount of siRNAs is produced and some may be able to reach the unprotected tissues. However, at this stage the viral infection is already established in scion tissues, the suppressor of gene silencing, HC-Pro is synthesised and these siRNAs cannot be efficiently used to combat the infection.

It is interesting to note that PTGS could efficiently spread from hpRNA rootstocks into the AS, but not into the WT scions. Although WT scions had accumulated less virus than the control grafts (WT scion grafted onto WT rootstock), their viral levels were still above the "non-infected" threshold. This phenomenon has been observed before by Palauqui *et al.* (1997) who noticed that co-suppression of nitrite reductase or GUS could only spread into scion tissues that were expressing the target transgene. These authors proposed that silencing in such scions is initiated when a transgene transcript accumulates beyond a threshold level. However, it is more likely that a limited amount of the silencing signal, containing siRNAs, reaches the scion tissues where the ability of the scion to amplify this signal determines whether effective silencing is established. If the scion transcribes a sequence homologous to the siRNAs, then siRNAs can be used as primers to amplify the target RNA thereby generating dsRNA and eventually siRNAs. As a result, effective silencing can be established in scions that contain a template (such as the AS transgene) for signal amplification. However, in scions that do not contain such a template (such as WT scions), the signal cannot be amplified and effective silencing is not observed. However, a small amount of siRNAs reaching the WT scions from the Hp rootstocks may help reduce viral levels, as observed in the WT scion / Hp rootstock grafts (Fig. 3.6 B).

The efficiency of systemic spread of silencing is also dependent on the sequence identity between the transgene from which the signal was derived and the target (i.e. challenging virus) that it encounters in the scion tissues. For example, the silencing signal sent from rootstocks expressing hpRNA transgene derived from PVY-D can effectively protect scions inoculated with the PVY-D strain, but not scions inoculated with the 55N strain (Fig. 3.7). This is somewhat unexpected as experiments described

in Chapter 2 established that tissues expressing a PVY-D derived hpRNA transgene are well protected against the 55N strain. Perhaps, because a limited amount of the signal reaches the scion, the differences in sequence become more important as, amongst the siRNAs comprising the silencing signal, there is only a small proportion of siRNAs that are able to target the 55N strain. However, if these siRNAs are also used to amplify the signal (using as a template either the transcript from the AS transgene or the viral RNA), then high levels of siRNAs able to target the virus will eventually be generated. Although this is likely to happen, by the time it does, the virus may be expressing HC-Pro and so suppressing the effectiveness of siRNAs. Although experiments described in this chapter were not able to test this hypothesis, further work analysing the levels of siRNAs and the dynamics of HC-Pro expression may offer some answers. In addition, experiments described in Chapter 4 attempt to establish whether an amplification step is an integral part of the silencing process.

Chapter 4

An investigation of the spread of gene silencing along the target molecule

4.1 Introduction

4.1.1 Aims

Transcriptional gene silencing (TGS) can be induced by hpRNA transgenes containing sequences homologous to promoter regions of the target gene. Small interfering RNAs (siRNAs) derived from hpRNA transgenes mediate methylation of cytosine residues within homologous DNA sequences leading to the establishment of TGS. It is currently not clear whether methylation induced by hpRNA transgenes remains confined to the targeted region, or spreads to the neighbouring sequences. Spread of methylation to the neighbouring sequences could affect expression of genes that were not originally targeted by the hpRNA construct.

Post-transcriptional gene silencing (PTGS) can also be induced by hpRNA transgenes. During PTGS, siRNAs derived from hpRNA transgenes guide the targeted cleavage of homologous RNA molecules and the methylation of cytosine residues in homologous DNA sequences. It has been proposed that siRNAs could also act as primers for amplification from a homologous RNA molecule. If the amplification step occurs, targeted degradation and methylation are likely to affect sequences flanking the originally targeted sequence. Determining whether the amplification step occurs is not only important for our understanding of the overall PTGS pathway, but also has significant practical implications for the design of constructs that aim to silence either a whole gene family or just one member of the family.

In Chapter 2 I suggested that the presence of an amplification step in a PTGS pathway could account for the ability of a transgene to establish an effective PTGS against a challenging virus that shares only 20nt of continuous sequence identity with the

transgene. However, an amplification step would also be likely to result in the spread of silencing, and methylation, along the target molecule. Experiments described in this chapter analyse methylation patterns associated with TGS and PTGS. These methylation maps are then used to determine whether TGS and PTGS spread from the originally targeted region to the flanking sequences.

4.1.2 Spread of silencing along the target molecule

The spread of silencing along the target molecule has been investigated using a variety of experimental approaches. The spread of TGS has been investigated only in the plant system while the spread of PTGS has been investigated *in vitro* using a *Drosophila* embryo extract and *in vivo* in *C. elegans* and in various plant experimental systems.

Aufsatz *et al.* (2002) investigated the spread of TGS in plants that contained two transgene constructs: 1) an NPT II gene conferring Kanamycin resistance driven by the NOS promoter; and 2) an inverted repeat derived from the NOS promoter sequence driven by the CaMV 19S promoter (Fig. 4.1). In this system, hpRNA produced by transcription of the NOS inverted repeat is processed into NOS siRNAs, which in turn mediate methylation of the NOS promoter sequence in the NOS:NPT II transgene (Fig. 4.1). Methylation of the NOS promoter leads to transcriptional silencing of the NPT II gene which renders plants sensitive to Kanamycin (Fig. 4.1). Although, the spread of methylation along the NOS promoter has only been investigated within a short distance (~50bp) from the originally targeted sequence, it appears that methylation remains largely confined to the directly targeted section.

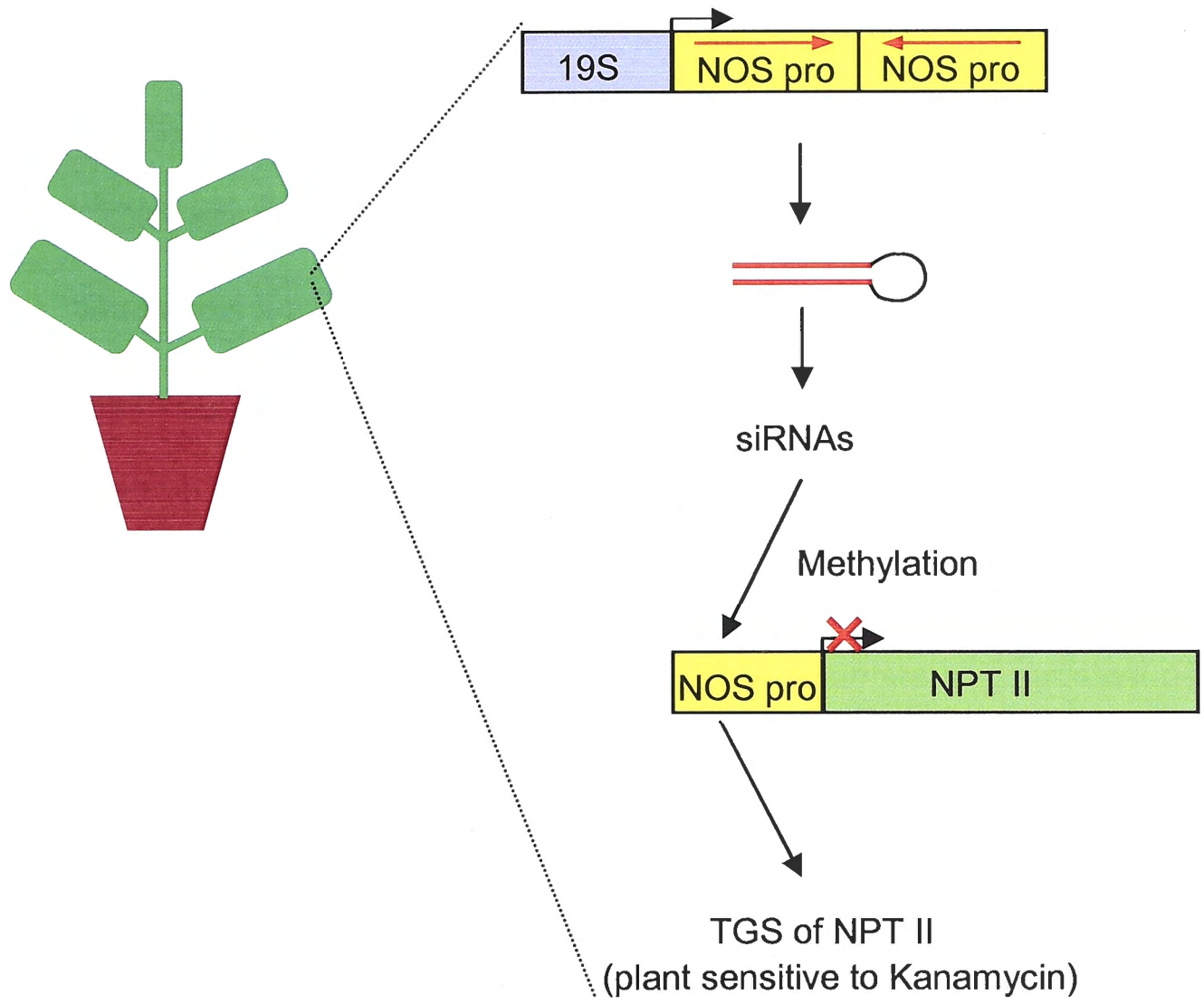


Figure 4.1: An experimental system used to study the spread of TGS. The 19S promoter drives an inverted repeat derived from the NOS promoter sequence. Transcripts derived from this construct produce hpRNA that are processed into siRNAs. These siRNAs guide methylation and subsequent transcriptional silencing of an unlinked NOS promoter that regulates the expression of the NPTII gene. When transcriptional silencing of the NOS:NPTII construct is established, plants become susceptible to Kanamycin.

An interesting study investigating the spread of PTGS along the target molecule was reported by Lipardi *et al.* (2001). The authors incubated green fluorescent protein (GFP) sense or anti-sense RNA molecules with radioactively labelled siRNAs derived from GFP in a *Drosphila* embryo extract (Fig. 4.2). After an incubation period, all single stranded RNA molecules were degraded by micrococcal nuclease digestion. The remaining dsRNA species were analysed by gel electrophoresis and transferred to a membrane, which was then used to expose a photographic film. The images revealed that the radioactively labelled siRNAs had been incorporated into dsRNA molecules. The size of dsRNA molecules was dependant on the incubation time, but could reach the full length of the GFP mRNA, 746bp. This suggested that siRNAs acted as primers and induced amplification from a single stranded RNA template to generate a dsRNA product. In subsequent experiments the authors demonstrated that these dsRNA molecules were later processed into siRNAs. Based on these observations the authors proposed a 'Degradative PCR' model for PTGS. The basic steps of 'Degradative PCR' model are outlined below:

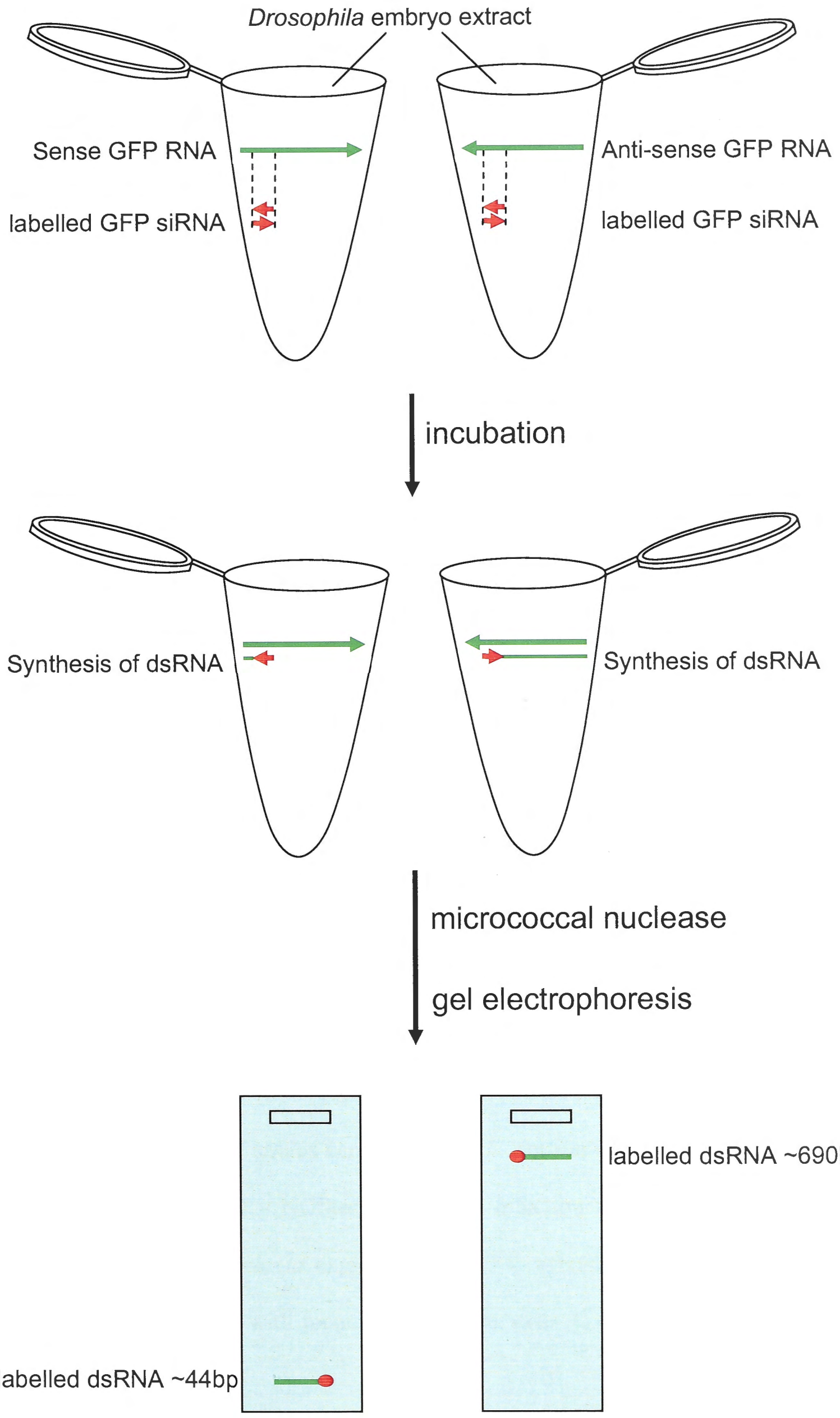
1. siRNAs act as primers for amplification from an RNA template;
2. Both sense and anti-sense RNA strands can act as templates for amplification;
3. Both strands of an siRNA molecule can act as primers;
4. Priming and subsequent extension from the primers generate dsRNA;
5. Newly generated dsRNA molecules are processed into secondary siRNAs.

The model put forward by Lipardi *et al.* (2001) has important implications for the spread of PTGS along the target sequence. According to this model, the amplification step can produce dsRNA molecules at least 700bp long. Furthermore, depending on the template (sense or anti-sense RNA) and the strand of siRNA being used as a

Figure 4.2: An investigation of the spread of PTGS using *Drosophila* embryo extract.

Green fluorescent protein (GFP) sense or anti-sense RNA molecules and radioactively labelled siRNAs derived from GFP were incubated in *Drosphila* embryo extract.

After an incubation period, all single stranded RNA molecules were degraded by micrococcal nuclease digestion. The remaining dsRNA species were analysed by gel electrophoresis and transferred to a membrane which was then used to expose a photographic film (Lipardi *et al.* 2001).


Drosophila embryo extract
Sense GFP RNA
labelled GFP siRNA
Anti-sense GFP RNA
labelled GFP siRNA
incubation
Synthesis of dsRNA
Synthesis of dsRNA
micrococcal nuclease
gel electrophoresis
labelled dsRNA ~44bp
labelled dsRNA ~690bp

primer, amplification could occur in either 5' or 3' direction. This effectively means that PTGS could spread at least 700bp, in either direction, from the originally targeted sequence.

A study by Sijen *et al.* (2001a) challenged the proposed long-distance spread of PTGS along the target molecule. This study used a phenotypic assay to investigate the spread of PTGS. The authors introduced a transgene composed of green fluorescent protein (GFP) and *unc*22Z sequences into *C. elegans*. PTGS of GFP was induced by injecting the transgenic worms with either full-length *GFP* dsRNA or dsRNA homologous only to a small section of GFP (Fig. 4.3). The appearance of a twitching phenotype, characteristic of *unc* silencing, indicated that silencing had spread from the originally targeted GFP sequence into the neighbouring *unc*22Z sequences (Fig. 4.3). Although the spread of PTGS was observed, it only occurred over a relatively short distance of ~120bp and could only spread into sequences positioned 5' from the original targeted area (Fig. 4.3). Furthermore, the spread of PTGS was dependant on an RNA dependant RNA polymerase (RdRP) homologue *rrf-1*, indicating that an RdRP may be the polymerase involved in the amplification step (Sijen *et al.* 2001a).

Vaistij *et al.* (2002) used virus induced gene silencing (VIGS) to investigate the spread of PTGS in a plant system. The authors inoculated plants expressing a GFP transgene with a recombinant tobacco rattle virus (TRV) variant that carried a portion of the GFP sequence. The recombinant TRV strains carried either a 5' portion of GFP (TRV:GF) or a 3' untranslated portion (TRV:NOSter) (Fig. 4.4). Infection with any of these strains resulted in PTGS of *in planta* expressed GFP. The spread of silencing was tested by secondary inoculation with recombinant potato virus X that carried the 3'

Figure 4.3: Assay used to investigate the spread of PTGS in *C. elegans* (adapted from Sijen *et al.* 2001)

A: Worms used in this assay carried transgenes encoding chimeric GFP/*unc*22Z or *unc*22Z/GFP transcripts. PTGS of GFP was induced when these worms were injected with either full length GFP dsRNA or dsRNAs derived only from a small section of the GFP gene.

B: The appearance of the twitching phenotype, associated with *unc* silencing, in worms injected with a GFP dsRNA was indicative of the spread of PTGS along the target molecule. Note that the spread of PTGS was only observed in the 5' direction and occurred over short distances.

Figure 4.4: A VIGS based system for investigation of the spread of PTGS.

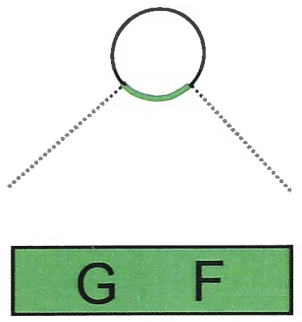
Plants expressing green fluorescent protein (GFP) were inoculated with recombinant tobacco rattle virus (TRV) variants. TRV variants carried either the 5' portion of the GFP gene (denoted as GF) or the 3' untranslated terminator sequence (denoted as NOSter).

Infection with either TRV:GF or TRV:NOSter result in PTGS of *in planta* expressed GFP.

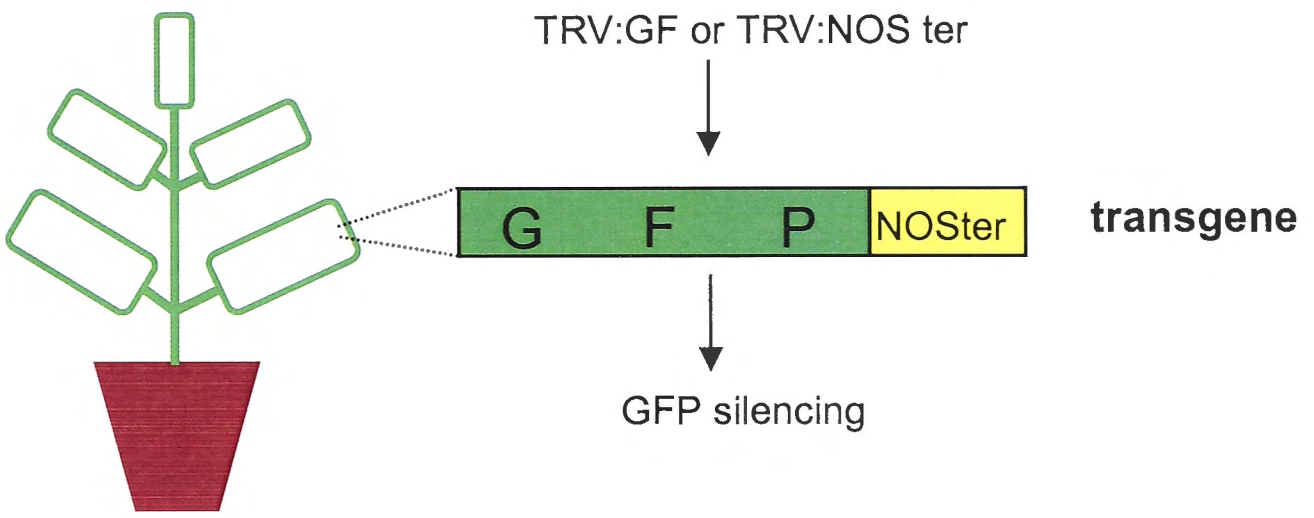
The spread of PTGS can be tested by inoculating GFP-silenced plants with recombinant potato virus X which carries the 3' portion of the GFP gene (denoted as PVX:P). Resistance to PVX:P would indicate that PTGS has spread from its original target (GF or NOSter sequences) into the adjacent P sequence (i.e. 3' region of the GFP gene).

Alternatively, the spread of PTGS can be investigated by analysing the identity of siRNAs present in GFP:NOS plants infected with TRV:GF or TRV:NOSter. The presence of siRNAs derived from the P section of GFP gene would indicate the spread of PTGS along the target molecule.

① **Inoculate GFP expressing plant with:**

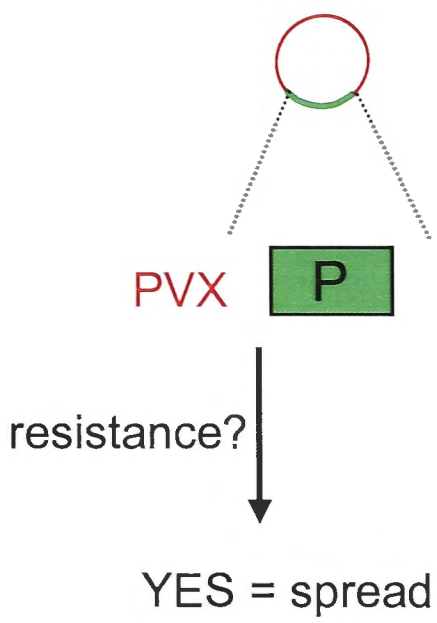


② **Establish GFP silencing in the plant that used to express GFP by virus infection**



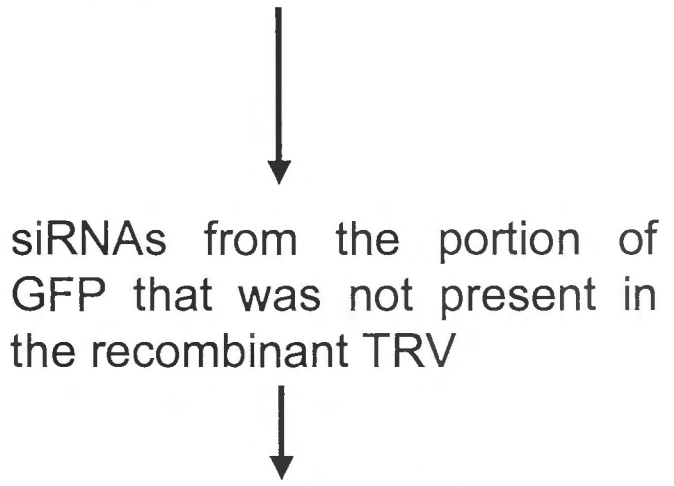
③ **Test for spread of PTGS by:**

ⓐ Performing secondary inoculation with:



OR

ⓑ Testing for siRNAs present in the GFP-silenced plants that were infected with TRV:GF or TRV:NOSter.



portion of GFP (PVX:P) (Fig. 4.4). Both plants inoculated with TRV:GF and plants inoculated with TRV:NOSter were resistant to PVX:P. This suggested that PTGS could spread both downstream (from the primary target, GF sequence, into P sequence) and upstream (from the NOS terminator sequence into P sequence). This finding was confirmed when siRNAs derived from the entire GFP sequence were detected in plants infected with TRV:GF or TRV:NOSter strains (Fig. 4.4). The sequence of siRNAs identified in this experiment suggested that PTGS can spread at least 332bp along the target molecule. In addition, consistent with previous reports and models, Vaistij *et al.* (2002) reported that the spread of PTGS was dependant on the putative RdRP SDE1/SGS2 and on the presence of the target transcript.

Another interesting finding reported by Vaistij *et al.* (2002) is that the pattern of DNA methylation mirrors the pattern of spread of PTGS. For example, if a plant expressing GFP is infected with TRV:GF then PTGS spreads from the primary target, GF, into the P section leading to production of siRNAs derived from the P portion of the gene. In such plants, both the directly targeted GF section and the adjacent P section of the GFP gene that is integrated in the plant genome are methylated. Furthermore, if the target gene was not transcribed then DNA methylation was confined to the primary target sequence. These findings support the proposal that siRNAs guide the methylation of homologous DNA sequences. Therefore, any spread of PTGS from the target sequence should also result in the spread of DNA methylation. As such, DNA methylation could be used as a reliable indicator for the spread of gene silencing.

4.1.3 The role of DNA methylation

Cytosine residues are methylated when a methyltransferase enzyme transfers a methyl group from S-adenosylmethionine (SAM) to the carbon-5 position of the pyrimidine ring of cytosine (Fig. 4.5). The *A. thaliana* genome contains at least ten genes that encode DNA methyltransferases. Based on their function, *A. thaliana* methyltransferases can be divided into three groups: MET, DRM (domain re-arranged methyltransferases) and CMT (chromomethylases) (Tariq & Paszkowski 2004).

The main function of the MET group of methyltransferases appears to be the maintenance of CG methylation. Mutations in MET1 lead to a loss of CG methylation, but have little effect on CnG (where n is A, C or T) and non-symmetric cytosine methylation. The second group of methyltransferases, DRMs, are responsible for de novo methylation of CG, CnG and non-symmetric cytosines while CMTs appear to be involved in the maintenance of non-symmetric cytosine methylation (reviewed in Tariq & Paszkowski 2004). All three families of methyltransferases are involved in RNA directed DNA methylation (RdDM) (Cao *et al.* 2003). DRMs are required for the establishment of RdDM in all sequence contexts, MET1 is involved in maintenance of CpG methylation while CMT3 and DRM maintain CnG and non-symmetric methylation (Fig. 4.6) (Cao *et al.* 2003).

Although there is currently no agreement about the details of the process, it is clear that RdDM plays a crucial role in the establishment and maintenance of TGS. The process is initiated by siRNAs that guide *de novo* DNA methylation activity of DRM methyltransferases (Cao *et al.* 2003). Methylated cytosine residues are recognised by proteins that have methyl cytosine binding domains (MBDs) (Fig. 4.6). These proteins

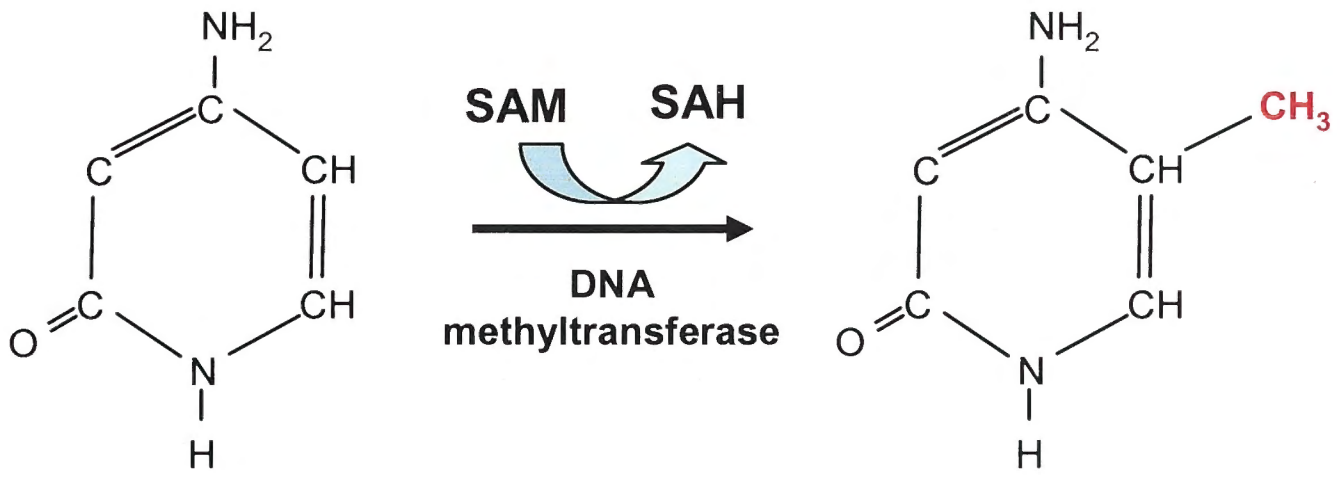


Figure 4.5: The conversion of cytosine to 5'-methylcytosine by a DNA methyltransferase.

The reaction involves the transfer of a methyl group from S-adenosylmethionine (SAM) to carbon-5 of pyrimidine ring of cytosine. During this process SAM is converted to S-adenosylhomocysteine (SAH).

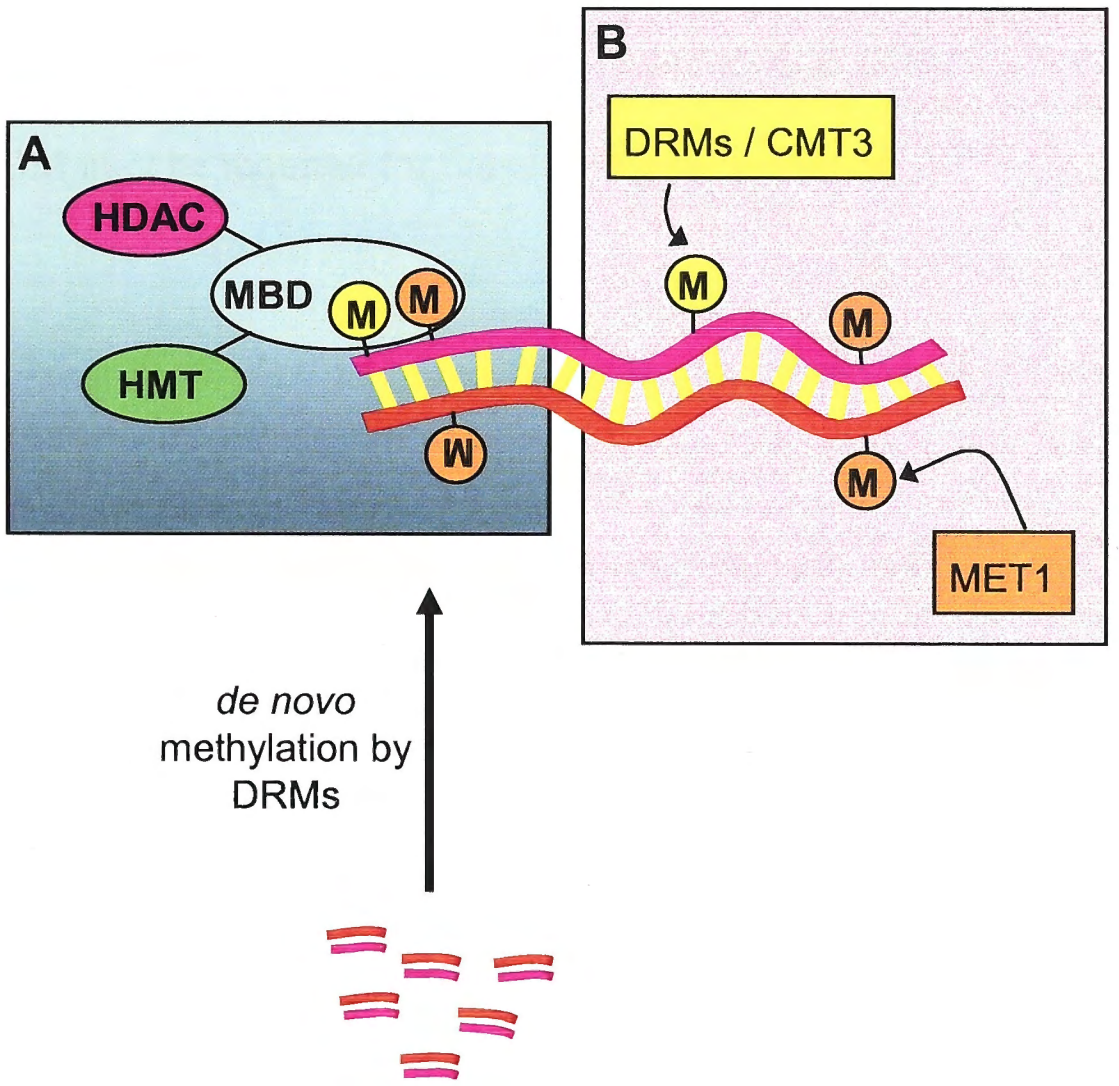


Figure 4.6: A simplified model of the establishment (A) and maintenance (B) of TGS in *A. thaliana*.

siRNAs guide DRM-mediated *de novo* DNA methylation. Proteins containing methyl binding domain (MBD) bind methylated DNA and recruit histone methyltransferases (HMTs) and histone deacetylases (HDACs). Histone methylation and deacetylation result in the establishment of heterochromatin. MET1 maintains metylation of CG cytosines (Ⓜ) while DRMs and CMTs maintain methylation of CnG and non-symmetric cytosines (Ⓜ).

are likely to recruit histone methyltransferases which act on the Lysine 9 residue of histone H3 (Fig. 4.6). These DNA and histone modifications result in the establishment of the inactive chromatin state (heterochromatin) and transcriptional silencing. MET1, CMT3 and DMRs act to maintain DNA methylation and the inactive chromatin state (Fig. 4.6).

In PTGS, siRNAs guide the degradation of both the target RNA and RdDM. Although the cleavage of the target RNA molecule is the key event in the PTGS mechanism, RdDM may also play an important role. For example, mutations in MET1 methyltransferase result in the stochastic release of PTGS during development (Morel *et al.* 2000). Furthermore, drug-induced hypomethylation of non-symmetric cytosines also results in partial release of PTGS (Kovarik *et al.* 2000). These findings suggest that RdDM may be required for the efficient maintenance of PTGS.

4.1.4 Using methylation maps to study the spread of gene silencing along the target molecule

In TGS, siRNAs guide the methylation of promoter sequences and initiate a cascade of events that lead to the establishment of heterochromatin. Any spread of TGS from the originally targeted region into the flanking sequences is expected to be marked by methylation of cytosine residues within these sequences. As such, a detailed analysis of methylation patterns within the target sequence and flanking DNA can provide reliable information about the spread of TGS along the target DNA molecule.

The spread of PTGS along the target molecule has been observed in several experimental systems (see Section 4.1.2). In this process, primary siRNAs derived

from the inducer of PTGS, such as a hpRNA construct, are likely to act as primers for RdRP-mediated amplification of the target RNA molecule. The end products of this amplification are secondary siRNAs which can be derived from sequences that are several hundred base pairs removed from the primary target sequence (Lipardi *et al.* 2001, Sijen *et al.* 2001a). Both primary and secondary siRNAs can guide methylation of cytosine residues within homologous DNA sequences (Vaistij *et al.* 2002). As such, the spread of RdDM from the targeted sequence into the flanking regions would indicate the presence of secondary siRNA species and the spread of PTGS along the target RNA molecule.

The methylation state of a DNA sequence can be examined using isoschizomers, enzymes that cleave the same target sequence but have a different response to its methylation status (Fig. 4.7). For example, enzymes *Hpa*II and *Msp*I both cleave the site CCGG. However, *Hpa*II cuts only when the second C is unmethylated, whilst *Msp*I cleaves the site irrespective of the methylation state of this C. *Msp*I can be used to identify all CCGG sequences within a given region and *Hpa*II can be used to determine the methylation state of CG cytosines within these sites. The size of DNA fragments derived from digestion with *Hpa*II and *Msp*I can compared and analysed for methylation patterns using gel electrophoresis, Southern blotting and appropriate imaging methods (Fig. 4.7). One important shortcoming of this experimental approach is that it does not provide information about the methylation state of numerous cytosines that lie outside sites targeted by the chosen restriction sites.

Bisulfite sequencing provides an alternate method for studying methylation and has the advantage of providing a map of the methylation status of every cytosine residue in

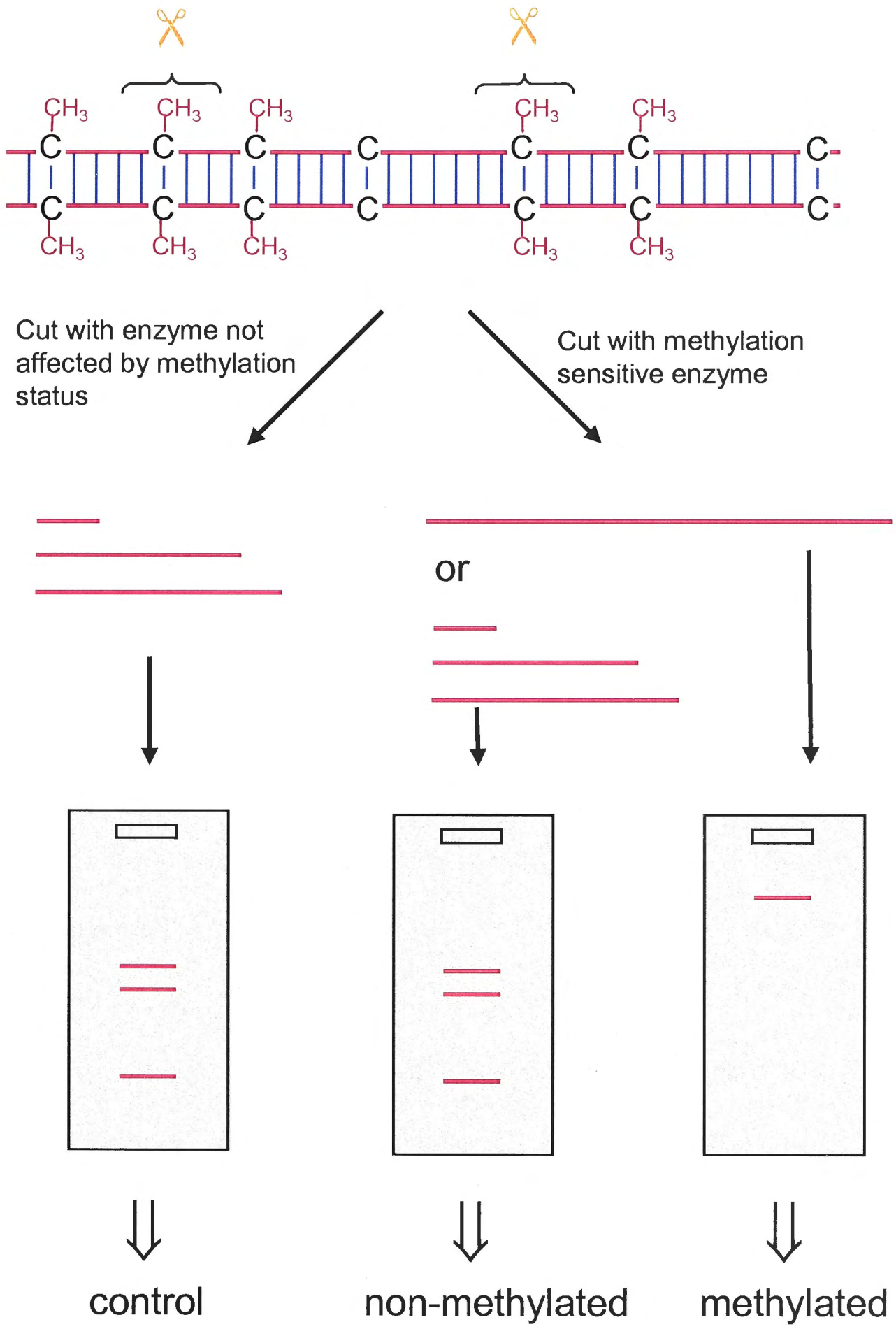


Figure 4.7: Analysis of cytosine methylation using methylation-sensitive isoschizomers.

A DNA sample is divided into two equal aliquots. The control sample is cut with an enzyme that is not affected by the methylation status. The test sample is cut with an enzyme that recognises the same DNA sequence, but is only able to cut if the DNA is not methylated. If the test sample produces the same band pattern as the control sample the DNA sequence is not methylated at the sites recognised by the enzymes used in the experiments. The presence of bands larger then those observed in the control sample is indicative of DNA methylation at the tested sites.

a particular region. During bisulfite treatment, cytosine residues are converted to uracil, while 5-methyl-cytosines remain unchanged (Clark *et al.* 1994). When PCR amplification products are sequenced, only cytosine residues that were methylated in the original genomic template remain, with unmethylated cytosines now appearing as thymine (Fig. 4.8).

4.1.5 Experimental strategy

The experiments described in this chapter were performed using several *A. thaliana* plant lines displaying TGS and PTGS of chalcone synthase gene (*CHS*).

TGS and PTGS were induced by a number of different hpRNA constructs targeting various regions of the *CHS* promoter and coding sequence respectively. Promoter regions targeted by the hpRNA constructs inducing TGS ranged from ~220bp to ~1000bp in length. Coding regions targeted by the hpRNA constructs inducing PTGS were either 100bp or 400bp in length.

I used a bisulfite sequencing approach to map cytosine methylation patterns within the *CHS* gene in *A. thaliana* plant lines carrying hpRNA constructs designed to induce TGS or PTGS. In the case of TGS, confinement of methylation to the sequences targeted by the hpRNA construct would suggest that methylation of a particular promoter region does not induce methylation and subsequent chromatin remodeling of large sections of DNA. In the case of PTGS, confinement of methylation to the area targeted by the hpRNA construct would indicate that siRNAs do not prime amplification of RNA templates and that the amplification step probably does not

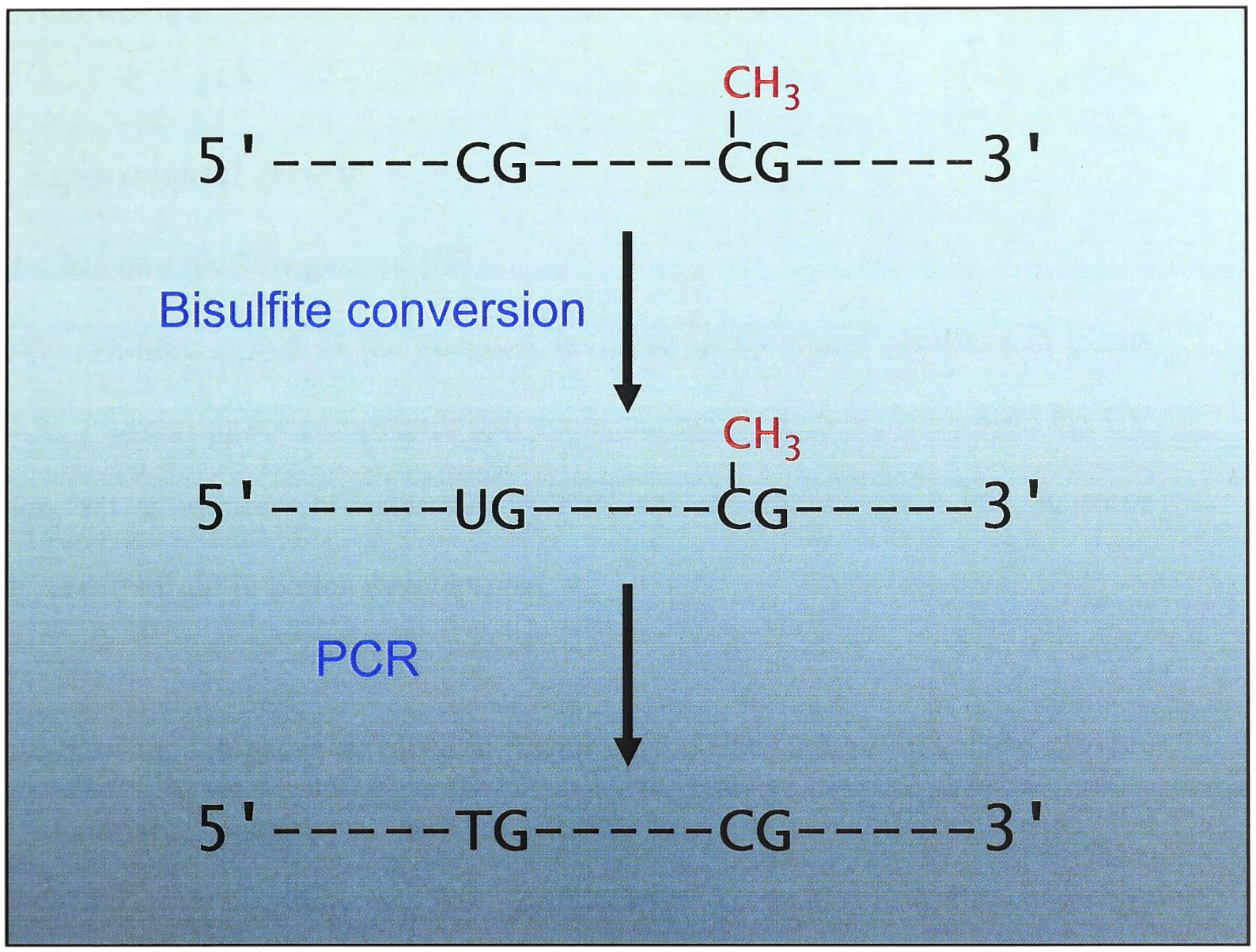
Figure 4.8: Bisulfite conversion and analysis of PCR products amplified from the bisulfite treated DNA .

A: Treatment of denatured genomic DNA with sodium bisulfite results in conversion of unmethylated cytosines to uracil. Methylated cytosines remain unchanged.

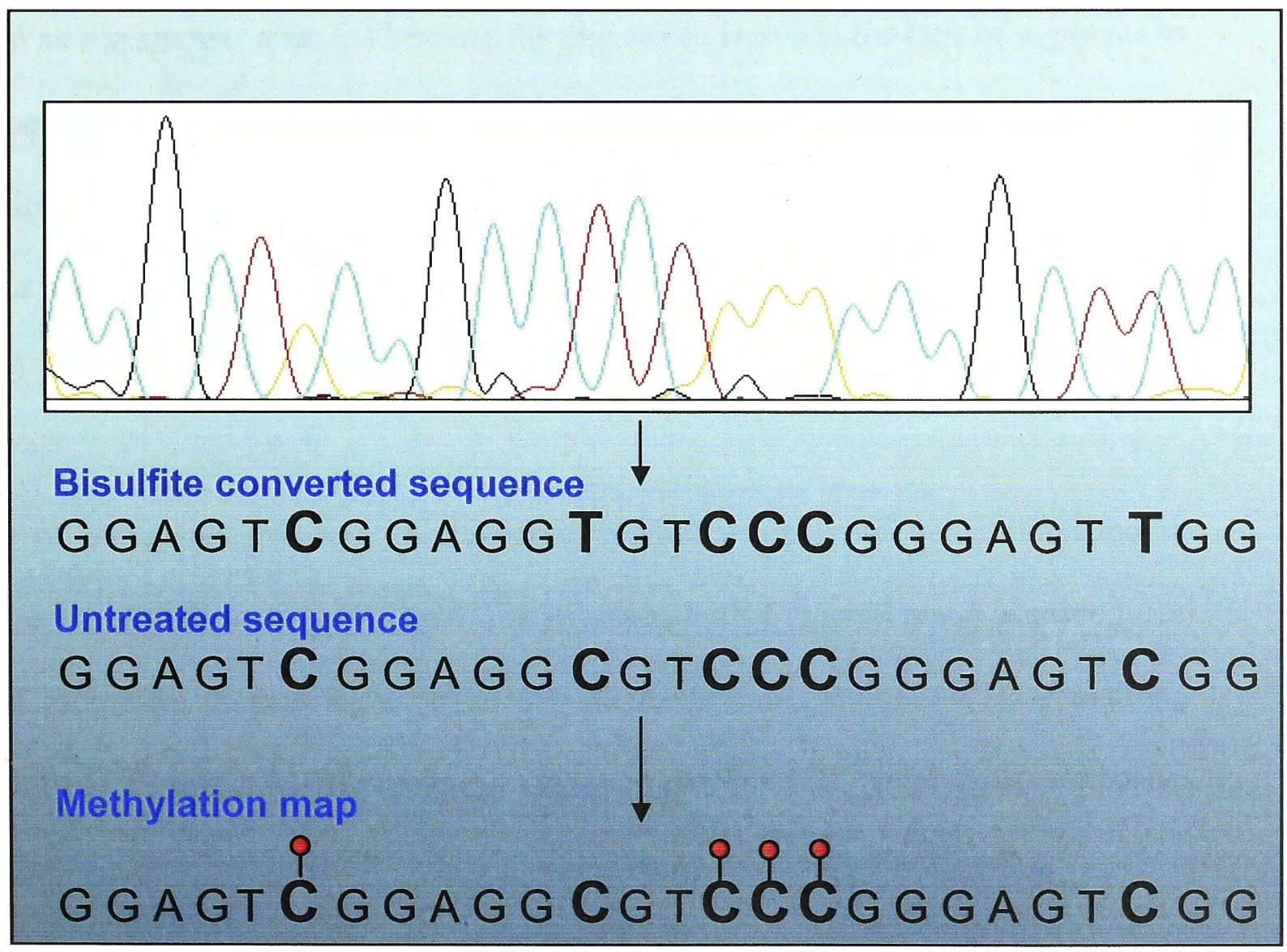
In the amplification process unmethylated cytosines in the starting DNA (now uracils) are replaced with thymines while the methylated cytosines remain as cytosines.

B: Comparison of the bisulfite treated sequence with the untreated sequence provides a methylation map of every cytosine residue (methylated cytosines marked with 🎈).

A


CH3
5'-----CG------CG-----3'
Bisulfite conversion
CH3
5'-----UG------CG-----3'
PCR
5'-----TG------CG-----3'

B


Bisulfite converted sequence
GGAGTCGGAGGTGTCCCGGGAGTTGG
Untreated sequence
GGAGTCGGAGGCGTCCCGGGAGTCGG
Methylation map
GGAGTCGGAGGCGTCCCGGGAGTCGG

occur. Furthermore, the observation that the spread of methylation occurs in PTGS but not in TGS would suggest that an RNA template is required for this type of spread.

4.1.6 Experimental system

4.1.6.1 Chalcone synthase gene (CHS)

Chalcone synthase is one of the enzymes involved in flavonoid synthesis in plants (Fig. 4.9). Flavonoids are compounds that act as pigments, protect plants from the UV radiation, act as inducers of bacterial virulence and nodulation genes and, in some species, are involved in pollen development.

Because mutations in genes involved in flavonoid synthesis are not lethal and provide easily identifiable phenotypes such as altered seed, stem or flower colour, the flavonoid synthesis pathway is well characterised. At least 11 loci required for flavonoid synthesis in *A. thaliana* have been identified. These loci are collectively known as *transparent testa* (*tt*) because the phenotype is due to the lack of pigments in the seed coat (testa) (Shirley *et al.* 1995). The seeds harvested from plants with mutations in the *tt4* locus, now identified as chalcone synthase, appear yellow or orange and are easily distinguishable for the brown seed produced by the wild type plants.

Some of the genes involved in flavonoid synthesis appear to be co-regulated in response to stimuli such as UV light. For example, both *CHS* and phenylalanine lyase (*PAL*) promoters contain light responsive elements such as the ACTG-containing element (ACE) and a MYB recognition element (MRE). The major light responsive

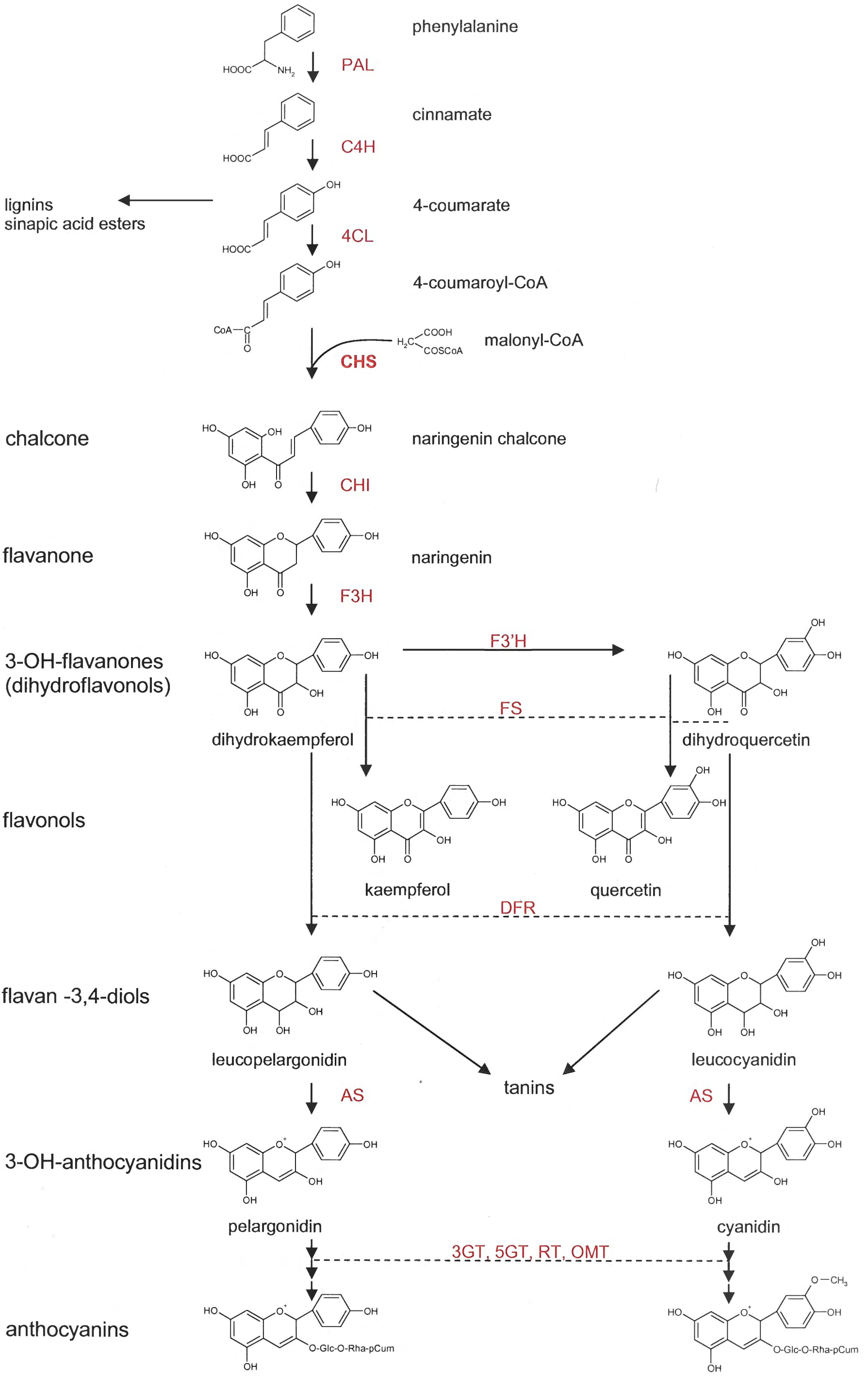


Figure 4.9: Flavonoid biosynthesis pathway (adapted from Sirley *et al.* 1995). Major enzymes involved in flavonoid biosynthesis are outlined in red.

region of the *CHS* promoter, located between -59 and -106, contains one ACE and one MRE element (Wingender *et al.* 1990, Hartmann *et al.* 1998) (Fig. 4.10).

4.1.6.2 Plants exhibiting TGS of CHS

I designed and generated three hpRNA constructs derived from the promoter region of *A. thaliana CHS* gene (Fig. 4.11). The three constructs were 998bp, 514bp, and 219bp in length. These constructs will be denoted as CHS1, CHS2 and CHS3 respectively.

CHS1, CHS 2 and CHS 3 constructs were transformed into *A. thaliana* plants. A large number of transformants were evaluated for *CHS* silencing by visual evaluation. For each of the CHS hpRNA constructs, the three *A. thaliana* plant lines with the most prominent *CHS* silencing phenotype were selected for use in the methylation mapping experiments.

4.1.6.3 Plants exhibiting PTGS of CHS

Plants expressing the 100bp and 400bp hpRNA constructs, derived from the coding region of *A. thaliana CHS* gene were generated and evaluated by Varsha Wesley (Wesley *et al.* 2001) (Fig. 4.11).

Plants expressing either the 400bp hpRNA transgene (denoted as CHS400 plants) or the 100bp hpRNA transgene (denoted as CHS100 plants) display a phenotype similar to that previously observed in *CHS* mutant (*tt4*) which was produced by ethyl methanesulfonate (EMS) treatment (Shirley *et al.* 1995, Wesley *et al.* 2001).

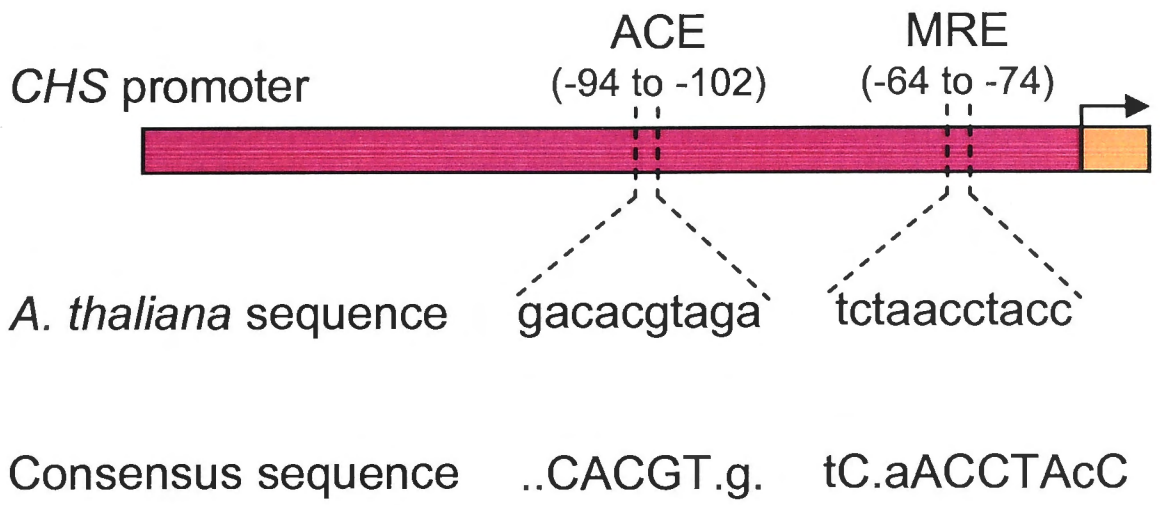


Figure 4.10: Location and sequence of two major light responsive elements present in the *A. thaliana CHS* promoter (adapted from Hartmann *et al.* 1998).

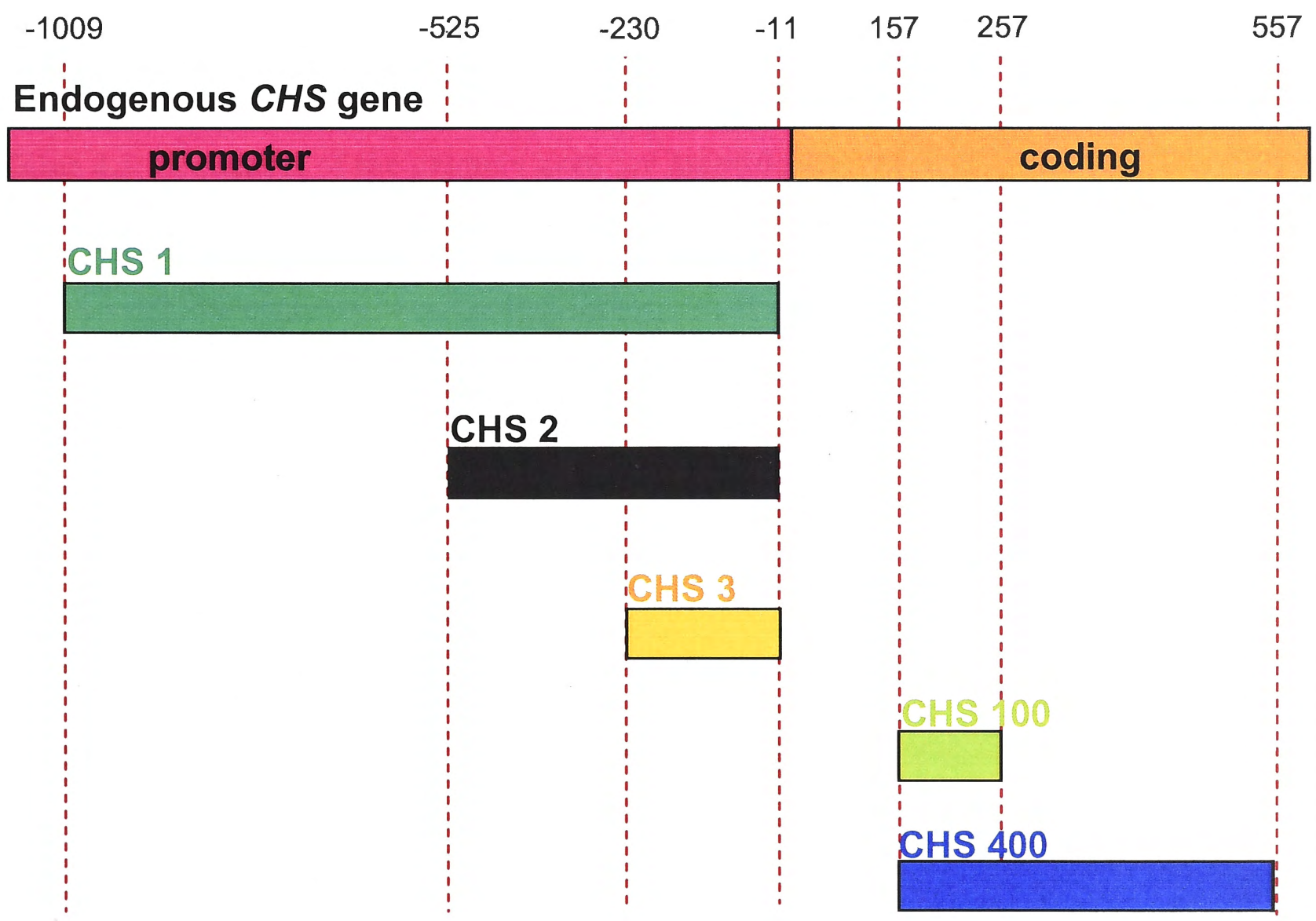


Figure 4.11: The position of the hpRNA constructs relative to the endogenous *CHS* gene.

CHS1, CHS2 and CHS3 hpRNA constructs have been derived from *A. thaliana CHS* promoter sequence. CHS100 and CHS400 have been derived from *A. thaliana CHS* coding sequences. All constructs are aligned parallel to the section of the endogenous *CHS* gene from which they were derived.

4.2 Materials and methods

4.2.1 Chalcone synthase hairpin (CHS Hp) constructs

The *A. thaliana* chalcone synthase promoter sequence was obtained from the TAIR database (accession no. At5g13930.1). Oligonucleotides used for amplification of the chalcone synthase promoter regions were designed using this sequence and information from the previous characterisation of the chalcone synthase promoter (Feinbaum & Ausubel 1988). Appropriate restriction sites were included in the primer sequence to facilitate cloning into a pHellsgate plasmid. Three promoter fragments of different sizes (CHS1=998bp, CHS2=514bp and CHS3=219bp) were amplified using the same reverse primer (CHS R1) in combination with one of the forward primers (CHS F1, CHS F2 or CHS F3). Primer sequences are given in Table 4.1.

Table 4.1: **Sequences of the primers used for amplification of *A. thaliana* chalcone synthase promoter fragments.** Italicised sequences represent introduced restriction sites.

Primer	Sequence
CHS R1 (Cla/Kpn)	ACA *ATCGAT* A *GGTACC* GAG AGT GAG AGC TTA TAT AAC AAA C
CHS F1 (Xba/Xho)	ACA *TCTAGA* A *CTCGAG* TCT ACG GCG TCT ACG CCT CGC ATG
CHS F2 (Xba/Xho)	ACA *TCTAGA* A *CTCGAG* GGG CCT AGT TAT AGG ATC ATA AGG
CHS F3 (Xba/Xho)	ACA *TCTAGA* A *CTCGAG* TTA ATA TGA GTT GTT GTT GTT GC

Polymerase chain reactions were prepared by adding 100ng *A. thaliana* (ecotype C24) DNA to a solution containing 1 × PCR buffer, 200μM PCR nucleotide mix, 0.3μM forward primer, 0.3μM reverse primer, 1.5mM $MgCl_2$ and 2 units of Taq polymerase in a final volume of 20μl. PCR buffer, nucleotide mix, $MgCl_2$ solution and Taq polymerase were supplied by Roche Applied Science (Germany)

Thermal cycling was performed as follows: 1 cycle of 95 °C / 3min, 35 cycles of 94°C / 15sec, 65°C / 15sec, 72°C / 1.5min, 1 cycle of 72°C / 3min and 1 cycle of 25°C / 5min.

5μl of each PCR product was electrophoresed on a 1.4% agarose gel. The presence of a band of the appropriate size (Fig. 4.12) indicated successful amplification. 100ng of each successful PCR reaction was used to subclone the PCR product into the plasmid pGEM-T Easy. Recombinant plasmids containing the desired inserts were identified and named pGEM-TE-CHS1, pGEM-TE-CHS2 and pGEM-TE-CHS3. CHS promoter fragments were then excised from the recombinant plasmids with either *Xho*I / *Kpn*I or *Xba*I / *Cla*I digestion (NBI Fermentas, Lithuania). All fragments were gel purified using a Qiagen Gel Extraction Kit (Qiagen, Germany).

The pHannibal plasmid (Wesley *et al.* 2001) was digested with the enzymes *Xho*I and *Kpn*I. The promoter fragments excised from the pGEM-T Easy vector by *Xho*I / *Kpn*I digestion were ligated with pHannibal (Fig. 4.13). The resulting plasmids were named pHan-CHS1sense, pHan-CHS2 sense and pHan-CHS3 sense (Fig. 4.13). Each of these plasmids was then digested with the restriction enzymes, *Xba*I and *Cla*I, and ligated with the appropriate CHS promoter fragment that had been excised from the pGEM-T Easy vector by *Xba*I and *Cla*I digestion. The resulting recombinant plasmids contained the full CHS hairpin constructs and were named pHan-CHS1Hp, pHan-CHS2Hp and pHan-CHS3Hp plasmids (Fig. 4.13). CHS hairpin constructs were excised by *Not*I digestion and introduced into the binary vector pART27 (Gleave 1992) (Fig. 4.13). The resulting recombinant plasmids were named pART27-CHS1Hp, pART27- CHS2Hp and pART27- CHS3Hp. To confirm the presence of the

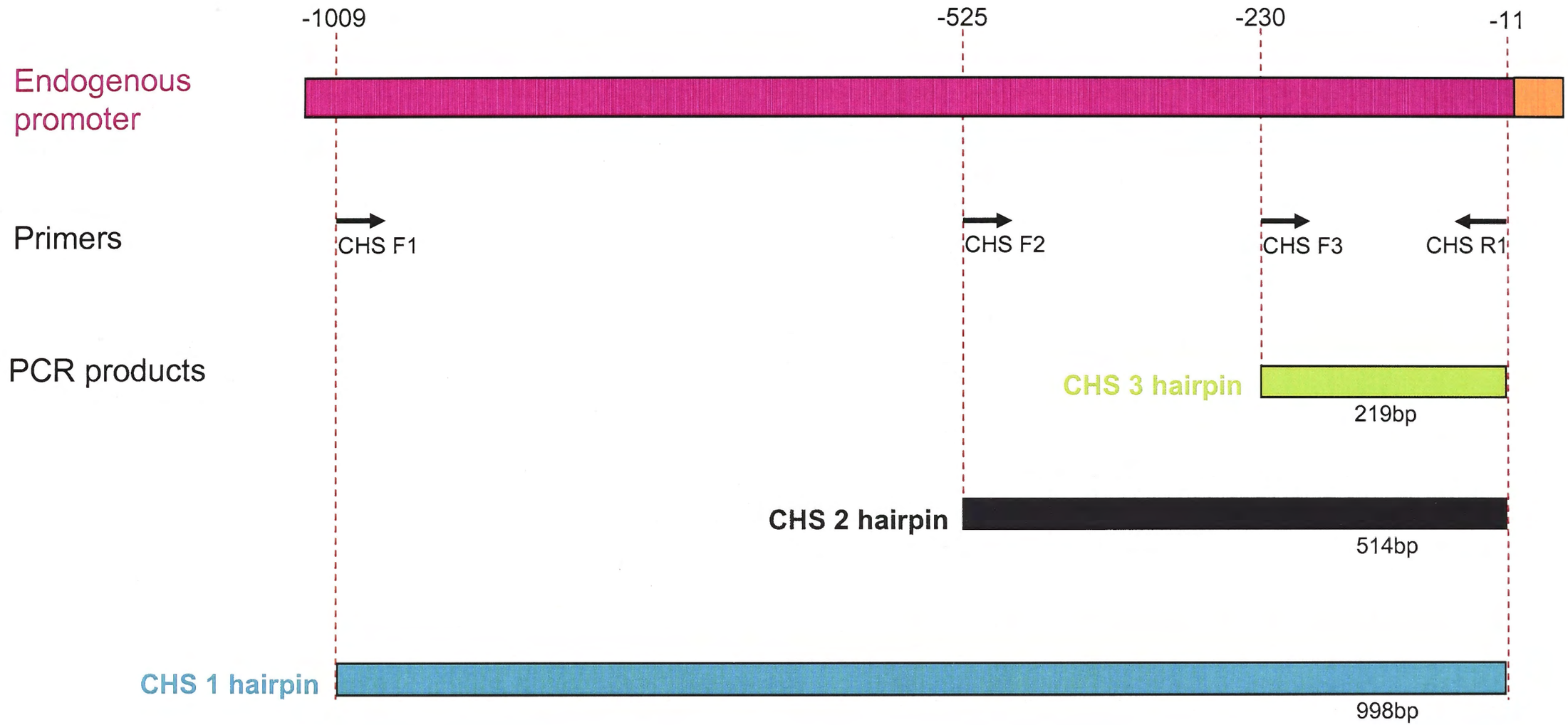


Figure 4.12: Positions of primers used for amplification of CHS1, CHS2 and CHS3 promoter fragments and the expected sizes of the amplification products.

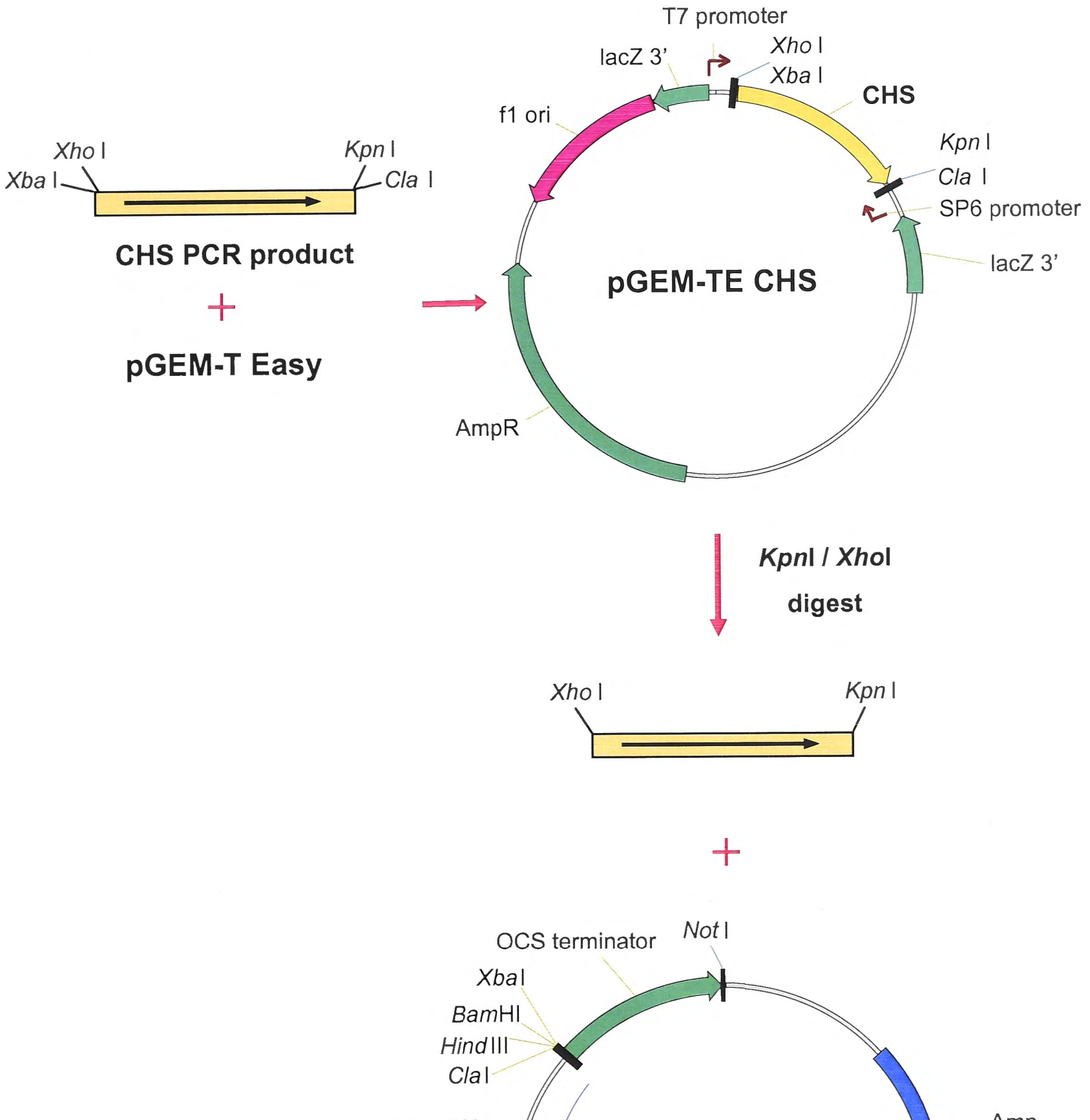
Figure 4.13: Cloning strategy used to generate CHS hpRNA constructs.

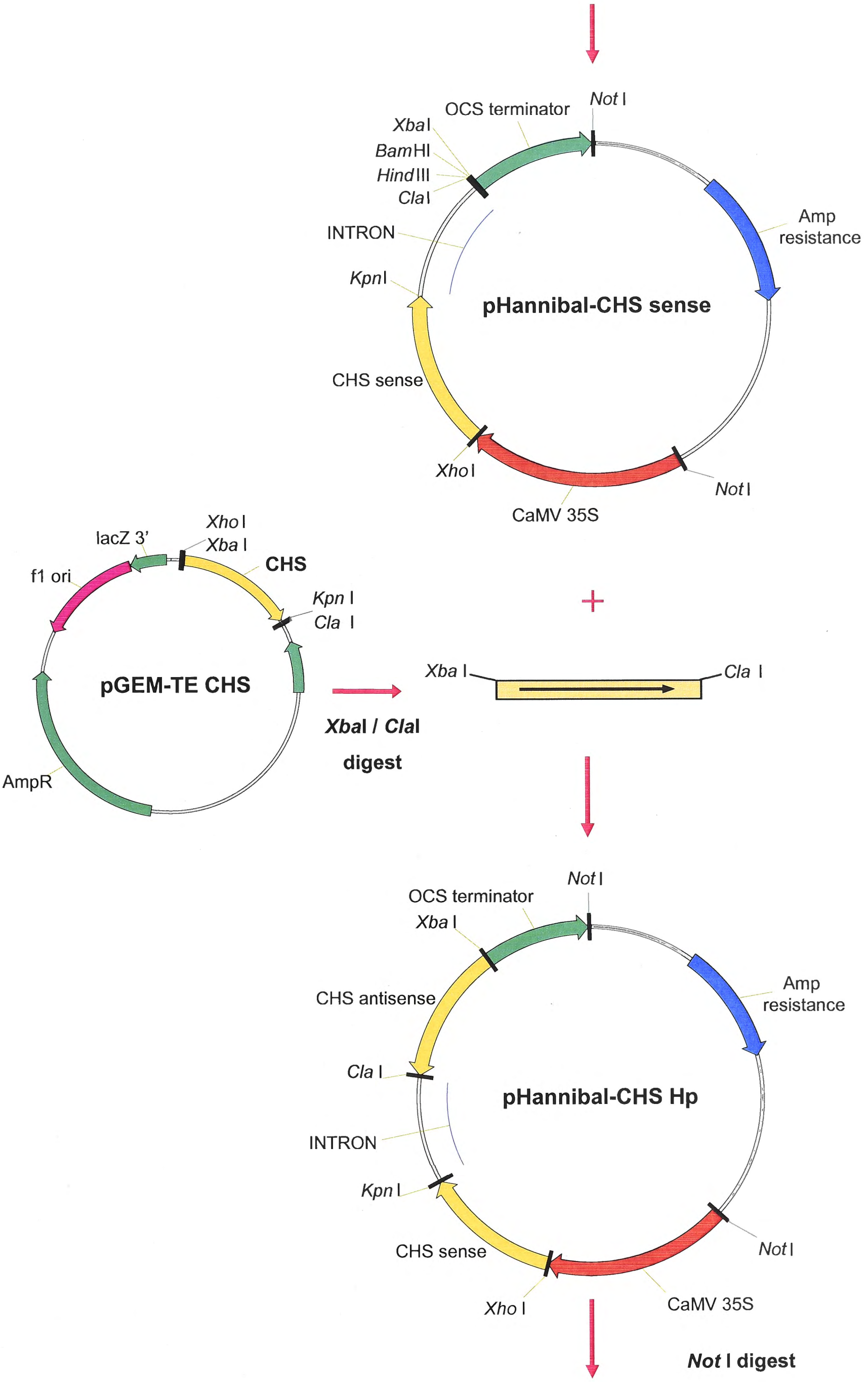
*Xba*I, *Xho*I, *Kpn*I and *Cla*I restriction sites were introduced into oligonucleotides used to amplify the appropriate fragment of the *CHS* promoter. The amplification product was subcloned into the pGEM-T Easy plasmid to generate a recombinant plasmid named pGEM-TE CHS.

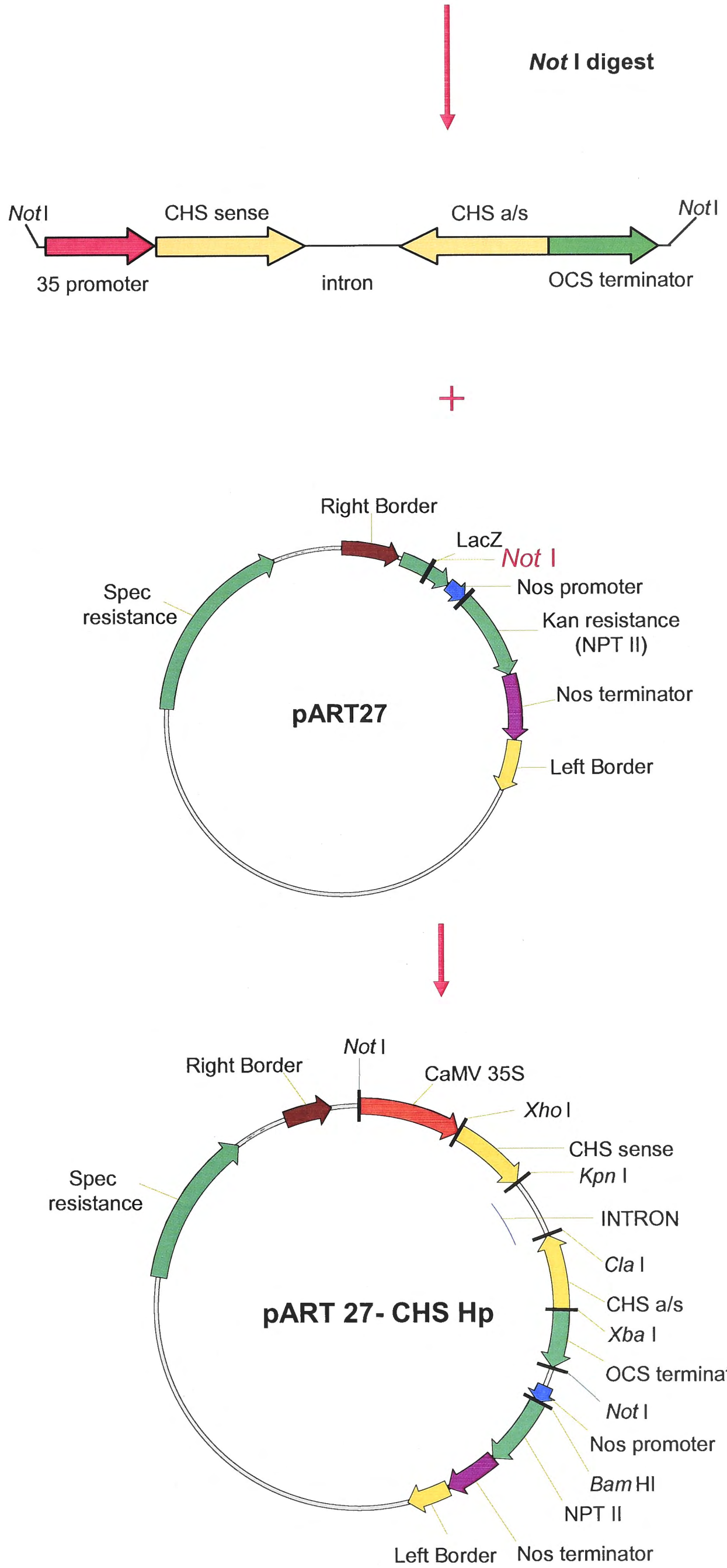
The *CHS* promoter fragment was then excised form the pGEM-TE CHS plasmid using *Kpn*I / *Xho*I digestion. This fragment was then subcloned, in sense orientation, into pHannibal to generate a recombinant plasmid named pHannibal-CHS sense.

The pHannibal-CHS sense plasmid was then digested with *Xba*I and *Cla*I restriction enzymes and ligated with the *CHS* promoter fragment that was previously excised from pGEM-T Easy with *Xho*I / *Kpn*I. The resulting recombinant plasmid contained the full CHS hairpin construct and was named pHan-CHS Hp.

The CHS hairpin construct was excised from pHan-CHS Hp with *Not*I digestion and introduced into binary vector pART27. The resulting recombinant plasmid was named pART27- CHSHp.


Xho I
Xba I
Kpn I
Cla I
CHS PCR product
+
pGEM-T Easy
T7 promoter
lacZ 3'
Xho I
Xba I
CHS
f1 ori
Kpn I
Cla I
SP6 promoter
lacZ 3'
pGEM-TE CHS
AmpR
KpnI / XhoI
digest
Xho I
Kpn I
+
OCS terminator
Not I
XbaI
BamHI
HindIII
ClaI
INTRON
Amp
resistance
pHannibal
KpnI
EcoRI
XhoI
CaMV 35S
Not I


OCS terminator
Not I
Xba I
BamHI
Hind III
Cla I
INTRON
Amp
resistance
Kpn I
pHannibal-CHS sense
CHS sense
Xho I
Not I
CaMV 35S
Xho I
lacZ 3'
Xba I
CHS
f1 ori
Kpn I
Cla I
pGEM-TE CHS
AmpR
Xba I
Cla I
Xba I / Cla I
digest
Not I
OCS terminator
Xba I
Amp
resistance
CHS antisense
Cla I
pHannibal-CHS Hp
INTRON
Kpn I
CHS sense
Not I
Xho I
CaMV 35S
Not I digest


Not I digest
*Not*I
CHS sense
CHS a/s
*Not*I
35 promoter
intron
OCS terminator
Right Border
LacZ
Not I
Nos promoter
Spec
resistance
Kan resistance
(NPT II)
Nos terminator
pART27
Left Border
Not I
Right Border
CaMV 35S
Xho I
CHS sense
Kpn I
Spec
resistance
INTRON
Cla I
CHS a/s
pART 27- CHS Hp
Xba I
OCS terminator
Not I
Nos promoter
Bam HI
NPT II
Left Border
Nos terminator

desired inserts, recombinant plasmids were re-digested with *Not*I and DNA fragments were separated on a 0.8% agarose gel. The presence of the correct sized bands (5.9kb for pART27-CHS1Hp, 4.9kb for pART27-CHS2Hp and 4.4kb for pART27-CHS3Hp) confirmed that the recombinant plasmids contained the desired inserts. Orientation of the inserts was determined following digestion with *Bam*HI and *Xba*I restriction enzymes. For all three recombinant plasmids this digestion yielded a 1.3kb fragment indicating that all three inserts had integrated in the +ve orientation (Fig. 4.13). Molecular cloning procedures were performed as outlined in section 2.2.3 and previously described by 2001.

pART27-CHS1Hp, pART27-CHS2Hp and pART27-CHS3Hp were electroporated into *Agrobacterium* GV3101 cells. Following electroporation the cells were streaked out on an LB plate (1% (w/v) tryptone, yeast extract 0.5% (w/v), NaCl 0.5% (w/v), 0.1% (v/v) 1M NaOH 1.5% agar) containing rifampicin (25μg/ml), gentomycin (25μg/ml) and spectomycin (50μg/ml). A single *Agrobacterium* colony was picked from each LB plate and used to inoculate 3ml of liquid LB media containing the same combination of antibiotics. The culture was grown at 28°C for 24 hours. The recombinant plasmids were re-isolated from 2ml of this culture and checked by restriction mapping to verify that the desired inserts were still present. The remainder of the bacterial culture was stored at 4°C and later used to prepare the bacterial culture that was to be used for *A. thaliana* transformation.

4.2.2 A. thaliana transformation

A. thaliana (C24 ecotype) seed was germinated on soil and allowed to grow until the primary inflorescence emerged. At this stage, bolts were clipped off and the plants were grown until secondary bolts formed buds.

0.5ml of *Agrobacterium* culture, known to contain the desired recombinant plasmid was used to inoculate 500ml of liquid LB media containing spectomycin (50μg/ml). The culture was grown overnight in a 28°C shaker, transferred into centrifugation bottles and centrifuged for 15 minutes at 5000rpm in a JA10 rotor (Beckman Coulter, USA). Following centrifugation, the supernatant was removed, the pelleted bacterial cells resuspended in 500ml dipping media (5% sucrose (w/v), 0.08% (v/v) Silwet-L77 (Lehle Seeds, USA)), and the solution transferred into a large plastic box.

Pots containing *A. thaliana* plants were inverted allowing the plant buds to be submerged in the bacterial solution. Following this, the plants were covered with plastic wrap and returned to the plant room. The plastic wrap was removed 2 days later. The dipping process was repeated 7 days later, and the plants were allowed to mature and set seed.

4.2.3 Plant growth in tissue culture and selection of transformants

A. thaliana plants were grown in 80mm petri dishes on Murashige-Skoog (MS) medium containing 3% sucrose and 0.8% agar, under 16 hour days with fluorescent lighting at ~75 μEinsteins, at 22°C. Plants carrying transgenes linked to the antibiotic resistance genes were selected by adding 50μg/ml of Kanamycin to the MS medium.

4.2.4 Visual evaluation of CHS silencing

Stems carrying mature siliques were cut from individual *A. thaliana* plants, placed in paper bags and allowed to dry for an additional two weeks. Seed was shaken off the dried stems and transferred into a fine strainer to remove other plant material. Seed colour was assessed visually under white and long-wave UV light (365nm). The primary transformants were classified into three categories (denoted as +, ++ and +++) according to their seed coat colour which reflected CHS activity (Fig. 4.14). Seeds that had very pale coat colour when observed under white light and a high degree of fluorescence when observed under UV light were given +++ classification. Seeds that did not show noticeable CHS silencing were denoted with '–'.

4.2.5 Extraction of plant RNA

100µg of *A. thaliana* tissue was ground in 1ml Trizol reagent (Invitrogen, US), incubated at RT for 5 minutes and centrifuged for 10 minutes at 13000rpm in a microfuge. The supernatant was transferred into a clean microfuge tube, 200µl chloroform was added and the tube was shaken by hand for 30 seconds. After centrifugation (4°C / 15min / 13000rpm) the aqueous layer was transferred into a clean microfuge tube, 0.5ml isopropanol was added and the mixture was incubated at RT for 10 minutes. RNA was pelleted by centrifugation (4°C / 15min / 13000rpm), the supernatant was removed and the pellet washed with 1ml 75% ethanol that was removed after another centrifugation step (4°C / 5min / 9300rpm). The pellet was allowed to dry for 5 minutes and finally dissolved in 20µl RNase-free water during a short incubation at 60°C. RNA was stored at -80°C.

4.2.6 Northern Blotting

Approximately 10 μg of each RNA sample was denatured at 65°C for 10 min in 50% (v/v) formamide, 17.5% (v/v) formaldehyde, 1 x N-morpholino propane-sulfonic acid (MOPS), 0.5 mg/ml ethidium bromide and separated on a 1.4% agarose gel containing 5% (v/v) formamide. The RNA was transferred to a nylon membrane by blotting in 20 × SSC. The membrane was pre-hybridised in 15ml Khandjian's solution (50mM Tris pH7.5, 1.5M NaCl, 50% formamide, 10× Denhardt's solution (2mg/ml ficoll, 2mg/ml polyvinylpyrrolidone, 2mg/ml bovine serum albumin), 10% dextran sulphate, 1% sodium dodecyl sulphate (SDS), 0.1% sodium pyrophosphate, 100μg/ml herring sperm DNA) at 42°C for 2 hours.

The 300 bp CHS fragment was cloned by reverse transcriptase-mediated PCR (RT-PCR) from *A. thaliana* leaf RNA using a One-step RT-PCR kit (Qiagen, Germany). The sequences of the forward and reverse primers used for amplification from the chalcone synthase transcript are outlined in Table 4.2. This 300bp chalcone synthase fragment was gel purified and used to synthesise a ^{32}P labelled DNA probe as outlined in 'Instructions for Renaissance Random Primer Extension Labelling System' (PerkinElmer Life Sciences, USA). The probe was denatured by heating at 100°C for 5 minutes and chilling on ice. The denatured probe was added to the hybridisation bottle containing the nylon membrane and 15ml of Khandjian's solution. Hybridisation proceeded for 16 hours at 42°C.

The membrane was washed with 2×SSC at room temperature, followed by 2×SSC, 0.1% SDS, 0.1% sodium pyrophosphate at 65°C for 15 minutes or until the overall background was less then 200cpm. The membrane was placed between plastic sheets,

sealed and put in a Phosphor-imager screen. ImageQuant software (v3.3; Molecular Dynamics) was used to visualise bands on the developed blots.

4.2.7 Reverse transcriptase polymerase chain reaction (RT-PCR)

100ng of RNA extracted from plant lines CHS1/3, CHS1/10, CHS1/15, CHS2/2, CHS2/8, CHS2/11, CHS3/17, CHS3/20 or CHS3/21 was used in each RT-PCR reaction. The reactions were prepared according to the manufacturer's instructions using a QIAGEN© OneStep RT-PCR kit (Qiagen GmbH, Germany). Each reaction was divided into two aliquots. Primers specific to the CHS gene were added to one of the aliquots, while primers specific to the 18S1 ribosomal unit were added to the second aliquot, which was used as an experimental control. Primers for amplification of *CHS* transcript were designed based on the Genbank cDNA sequence for chalcone synthase (accession no. BT000596) (Table 4.2). Primers described in Klok *et al.* (2002) were used to amplify the 18S1 transcript.

Table 4.2: **Sequences of primers used for RT-PCR of chalcone synthase transcript**. Italicised sequences represent introduced restriction sites.

Primer	Sequence
CHS F (133) CHS R (143)	*CTGCAG* GCA CTG CTA ACC CTG AGA ACC *GGTACC* TTG ACT TGG GCT GGC CCC ACT

4.2.8 Thin layer chromatography (TLC)

T2 plants carrying CHS1, CHS2 or CHS3 transgenes were selected by growing the seed on a Kanamycin containing medium. 20mg of seed collected from at least 5 T2 plants from each analysed line was ground into a fine paste using a mortar and pestle.

The paste was resuspended in 1ml of 1% HCl in methanol, transferred into a 10ml falcon tube and left at 4°C for 16 hours. The crude anthocyanin preparations were extracted further using Folch partitioning (Folch 1951) with chloroform/ H_2O to remove chlorophyll and then extracted with hexane. To simplify the interpretation of chromatograms, the glycosides were removed by acid hydrolysis and the free aglycones examined. Samples were hydrolysed by adding an equal volume of 37% HCl and boiling for 15 min. Boiled samples were then extracted into pentan-2-ol, which was evaporated under vacuum centrifugation. Samples were dissolved in 1% HCl in methanol, spotted onto 0.1 mm cellulose TLC plates (Merck), and developed using A & F #9 (HCl: formic acid: H_2O 19: 40: 41 v/v/v) (Andersen and Francis, 1985). Dried plates were sprayed with 1% methanolic diphenylboryloxyethylamine (NP stain), followed by 5% ethanolic polyethylene glycol 4000 and then analysed for anthocyanins and flavonols. Images of the plates were recorded in visible light with an HPScanJet 4C/T scanner or photographed under UV illumination at 365 nm.

4.2.9 High performance liquid chromatography (HPLC)

Anthocyanins were extracted as described in section 4.2.8. After extraction with hexane, 200µl of the extract was placed in a clean microfuge tube and dried in a heated vacuum centrifuge. Lyophilised anthocyanins were resuspended in 100µl MQ water and loaded into HPLC vials. The samples were analysed by HPLC on an Activon (Australia) Goldpack 3 cm x 0.46 cm (ID) column packed with 3µ Exsil 100A, ODS C18 packing and eluted at 2ml/min with a gradient from solvent A (2% v/v aqueous acetic acid) to 60% solvent B (methanol) over 10 min, and returning to starting conditions over 5 min, with the detector set at 280 nm. The void volume of the column and system was 500 µL.

4.2.10 Extraction of plant DNA

1.5 g of *A. thaliana* tissue was ground in liquid nitrogen and resuspended in 5ml hot (65°C) CTAB buffer (140mM Sorbitol, 220mM Tris pH 8.0, 22mM EDTA pH 8.0, 800mM NaCl, 1% Sarkosyl, 0.8% CTAB (w/v), pH 8.0). After a 20-minute incubation at room temperature (RT), 2.5ml chloroform was added to the mixture and incubated, with occasional shaking, for additional 20 minutes. Following this incubation, the mixture was centrifuged at 4000g for 10 minutes. The aqueous phase was removed, mixed with 3.5ml isopropanol, placed on ice for 10 minutes and centrifuged at 3500 rpm for another 10 minutes. The precipitate was resuspended in 0.5ml TE buffer, pH 8.0 (0.1 mM EDTA pH8.0, 10mM Tris pH 7.5) and transferred into a 1.5ml microfuge tube. 0.5ml chloroform and 0.5 ml 2xCTAB buffer (2% CTAB (w/v), 1.4M NaCl, 100mM Tris pH 8.0, 200mM EDTA pH 8.0, 1% PVP) were added and the tube vortexed for 30 seconds. The mixture was centrifuged at 13 000 rpm in a bench centrifuge for 10 minutes. The aqueous phase was removed and mixed with 50 µl of 3M sodium acetate (CH_3COONa) and 1ml cold ethanol. The mixture was incubated on ice for 15 minutes and a DNA pellet was formed after centrifugation in a bench centrifuge (10 minutes, 13 000 rpm). The pellet was washed with cold 70% ethanol, dried and finally resuspended in 100µl TE buffer (pH 8.0).

4.2.11 Analysis of DNA methylation

4.2.11.1 Bisulfite treatment

MQ water was added to 4µg genomic DNA to bring the final volume to 100µl. 11µl of 3M NaOH was added to the DNA solution, mixed and incubated for 15 minutes at 37°C. Following incubation, 1ml of 0.3M sodium bisulfite (Sigma, USA) and 60µl of

0.1mM hydroxyquinone were added to the DNA. The mixture was covered with paraffin oil and incubated at 55°C for 16 hours.

4.2.11.2 Salt removal

Following bisulfite treatment the paraffin oil was removed and each reaction was split in two 500µl aliquots. To remove salts, each aliquot was treated with Wizard DNA clean-up resin (Promega, USA) according to manufacturer's instructions. Purified aliquots originating from the same reaction were then combined bringing the total volume to 100µl. 11µl of 1.2M NaOH was added to the DNA solution and the reaction was incubated at 37°C for 15 minutes. To precipitate the DNA 12µl sodium acetate (3M, pH 5.5) and 400µl cold ethanol were added to the solution, the mixture was vortexed and placed at -20°C for 2 hours. The DNA was pelleted by centrifugation at 13000 rpm for 15 minutes, the aqueous solution removed and the DNA pellet resuspended in 100µl MQ water.

4.2.11.3 Polymerase chain reaction (PCR)

PCR primers were designed following the method outlined by Clark *et al.* 1994. Sequences of primers used for methylation analysis are given in table 4.3.

To increase specificity of amplification a "Hot start" PCR approach was used. This approach involves the separation of the DNA template and the Taq polymerase from other components until the reaction reaches approximately 90°C. To achieve this, the PCR reaction was divided into two portions that were separated by a layer of wax that melted and allowed these components to mix as the temperature approached 90°C.

Table 4.3: Sequences of the primers used for analysis of methylation of the promoter and coding regions of the *A. thaliana CHS* gene.

Primer	Sequence
BisCHS F1	TTT TTT GTA TGG TGA AGA AAT TAT ATA AT
BisCHS F2	TTA TAG TTG ATT AAA TAA TAT TTA TAG GT
BisCHS F3	TTT ATG TGG GTT TAT GGT ATA ATT TTT TT
BisCHS F4	TGA AAA ATA AAA TAA ATG TAT TTT GGT ATT
BisCHS F5	TTT GTG TTA GTT TGT TAT ATA AGT TTT TAT TT
BisCHS F6	GGA GTA TTT TGA TTA TTA TTT TTG TAT TAT T
BisCHS R1	ATA TAC TAA ACA AAT TAA ATA TAT TTA A
BisCHS R2	TAA TAT TCA TCA CAT TCT TTT TAA TAT ATA
BisCHS R3	TAA CTA CTA ATT AAA TAA TAT TAC ATT AAA
BisCHS R4	AAA TAA AAT ATA TAT TTA TTA TAT CTT AAA
BisCHS R5	ACC AAA AAA AAT AAA AAT AAA AAT TAA TAA A
BisCHS R6	AAC TTA AAC TTA CCC CAC TCC TTA ATA A

The "bottom" portion of the PCR reaction was assembled in a 200µl microfuge tube and contained 2.5µl 10 x PCR buffer, 10µl 25mM $MgCl_2$, 5µl of 10µM forward primer, 5µl of 10µM reverse primer, 2.5µl 10mM dNTPs and one wax bead (Perkin Elmer, USA). The reaction was incubated at 80°C for 5 minutes in a PCR machine and then cooled at 4°C for an additional 5 minutes. This treatment caused the wax bead to melt and re-solidify forming a seal over the bottom portion of the PCR reaction. The "top" portion of the PCR reaction containing 5µl of bisulfite treated DNA, 1µl Amplitaq Gold polymerase (Perkin Elmer, USA), 10µl 10xPCR buffer and 59µl MQ water was then placed on the wax seal.

Thermal cycling was performed as follows: 1 cycle of 94 °C / 12min, 5 cycles of 94°C / 1 min, 50°C / 2.5 min, 72°C / 3min, 30 cycles of 94°C / 30sec, 50°C / 1.5min, 72°C / 1.5min, 1 cycle of 72°C / 10min and 1 cycle of 25°C / 5min.

4.2.11.4 Subcloning of bisulfite PCR products

PCR products were ligated into the pGEM-T Easy vector (Promega, USA) and electroporated into *E. coli* DH5α cells as described in section 2.2.3. Transformed cells were plated out on an LB plate (1% (w/v) tryptone, yeast extract 0.5% (w/v), NaCl 0.5% (w/v), 0.1% (v/v) 1M NaOH 1.5% agar) containing Ampicillin (100μg/ml), X-Gal (50μg/ml) and IPTG (25μg/ml). The plates were incubated at 37°C for 16 hours. The use of the pGEM-T Easy vector allowed for blue/white colony selection. A white colony should contain a recombinant plasmid while a blue colony should contain an empty plasmid.

4.2.11.5 Sequencing of subcloned inserts

TYGPN medium (2% (w/v) tryptone, 1% (w/v) yeast extract, 1% (v/v) 80% glycerol, 0.5% (w/v) Na_2HPO_4, 1% (w/v) KNO_3, 100μg/ml Ampicillin) was aliquoted into 96-well microtiter plates (150μl / well) and each well was inoculated with one white bacterial colony. The plates were sealed and incubated for two days at 37°C. Approximately 3μl of the final culture was used as a template for a PCR reaction using 20 pmol universal forward (GTA AAA CGA CGG CCA GT) and reverse (AAC AGC TAT GAC CAT G) sequencing primers. All PCR reactions were performed in 96-well plates (AB gene, UK). The PCR conditions were as follows: 35 cycles of denaturing at 95°C for 30s, annealing at 52°C for 30s and extension at 72°C for 1 min. The PCR products were examined on 1.5% agarose gel and purified using ethanol precipitation. DNA pellets were resuspended in 50μl of 10mM Tris (pH 8.0). 1μl of a DNA resuspension was used as a template for a DNA sequencing reaction. All sequencing reactions were performed using Big Dye Terminator 3.1 chemistry (Applied Biosystems, USA) and primed using a reverse sequencing primer. Conditions for

sequencing reactions were as follows: 25 cycles of denaturing at 96°C for 10s, annealing at 50°C for 5s and extension at 60°C for 4 min. Excess dye terminators were removed using ethanol precipitation. Dye-labelled products were gel separated and sequenced at the Australian Genome Research Facility (AGRF) using 3730xl automatic capillary DNA sequencer (Applied Biosystems, USA).

4.2.11.6 Sequence analysis

Sequencing trace files were analysed using web-based Bio-Manager software provided by the Australian National Genomic Information Service (ANGIS).

4.3 Results

4.3.1 Evaluation of efficiency of transcriptional silencing of CHS gene

The efficiency with which the *CHS* gene was silenced was evaluated using both visual and molecular approaches. The first step was to evaluate silencing visually by examining the colour of T2 seed (Fig. 4.14) under white light and long wave UV light. A total of 17 CHS1, 20 CHS2 and 36 CHS3 plant lines were assessed for seed coat colour. Seeds displaying CHS silencing were classed into three categories, denoted as +, ++ and +++ (Fig. 4.14). Seeds that had a very pale coat colour when observed under white light and a high degree of fluorescence when observed under UV light were given +++ classification, while seeds that showed no silencing were denoted as '–'. Seeds from several plant lines did not appear to have the silencing phenotype when observed under white light although they displayed some fluorescence when observed under a more sensitive UV light. Visual evaluation scores given to *A. thaliana* lines

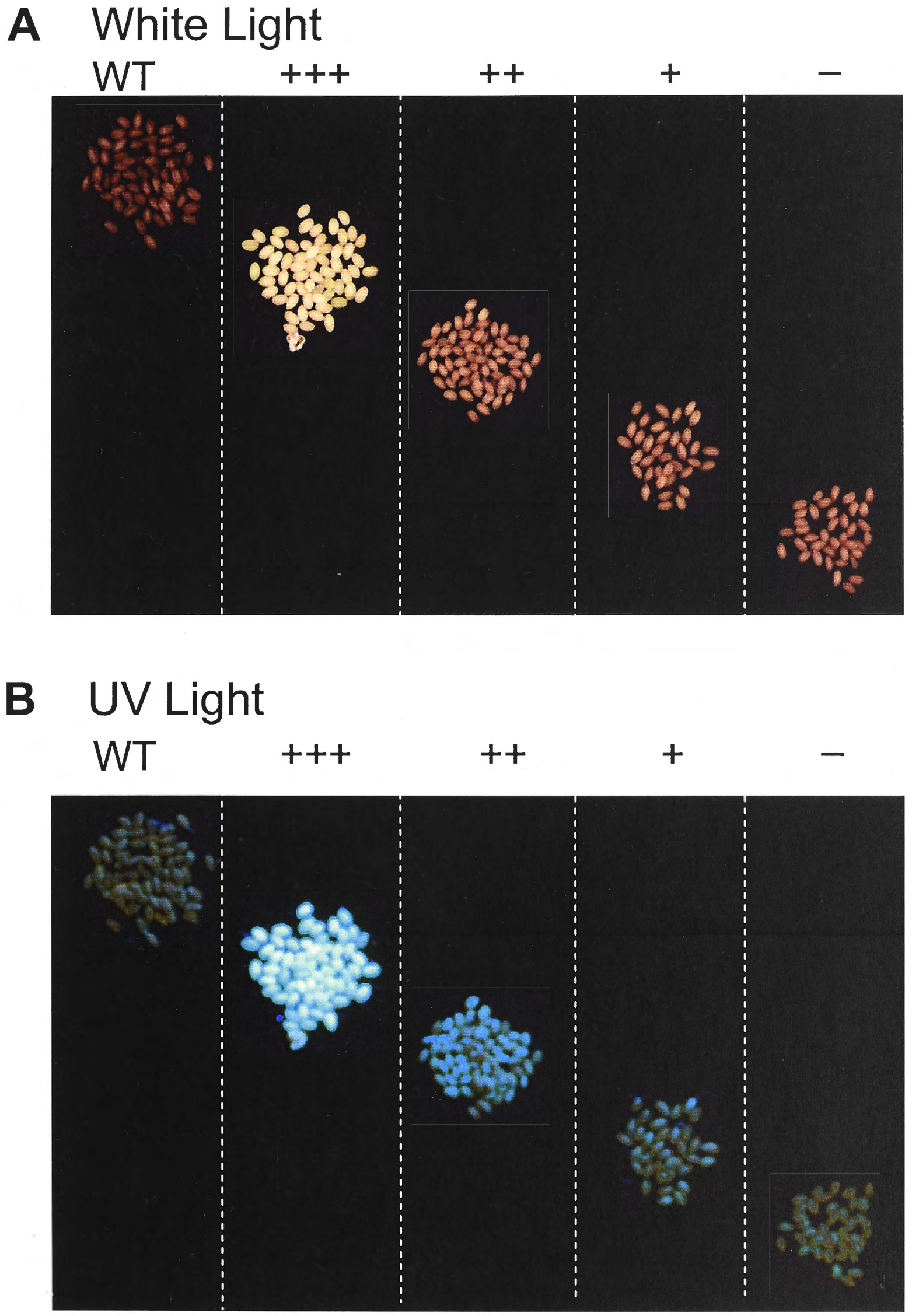


Figure 4.14: Visual evaluation of transcriptional silencing of the *CHS* gene. Seeds displaying most prominent silencing are denoted with '+++', those displaying progressively weaker silencing are denoted with '++' or '+', while those not showing significant silencing are marked with '-'.

carrying constructs for transcriptional silencing of the *CHS* gene are outlined in Table 4.4.

A plant line was considered to display significant chalcone synthase silencing if its T2 seed was given at least a '++' score for each of the visual evaluations (white light and UV light). 18% of plant lines carrying the CHS1 construct for transcriptional silencing of *CHS* displayed significant silencing while 15% of plant lines carrying the CHS2 construct and 39% of plant lines carrying the CHS3 construct displayed a significant degree of silencing. One plant line, CHS2/2 (*CHS* silencing construct 2 / line 2), displayed a particularly striking silencing phenotype – under white light its seed coat was light yellow in colour and under UV light its seed had a very high level of fluorescence. These observations suggest that the shortest transcriptional silencing construct, CHS3, was the most consistent in inducing *CHS* silencing. However, the most complete *CHS* silencing was observed in a plant carrying the CHS2 construct indicating that the length of the construct may not be the most important factor in determining the efficiency of silencing. To further investigate the characteristics of transcriptional silencing in this system a total of 9 plant lines, 3 lines with highest visual evaluation scores for each of the *CHS* silencing construct, were selected for further analysis.

In order to establish whether the chosen plant lines contained single or multiple copies of the transcriptional silencing constructs, T2 seed from each of the 9 plant lines (CHS1/3, CHS1/10, CHS1/15, CHS2/2, CHS2/8, CHS2/11, CHS3/17, CHS3/20 and CHS3/21) were germinated on a medium containing Kanamycin. The Kanamycin resistance gene (NPTII) was on the same transfer DNA (T-DNA) fragment as the *CHS*

Table 4.4: Scores for visual evaluation of transcriptional *CHS* silencing.
Score '+++' denotes most prominent silencing, scores '++' and '+' denote progressively weaker silencing while '–' denotes no observable silencing. Seeds that did not clearly belong to one of the categories are denoted with scores for the two closest categories (for example: '++ / +++'). Plant lines selected for further analysis are printed in red font.

CHS1	Plant no.	White Light	UV Light
	1	+	–
	2	+	–
	3	++ / +++	++
	4	+	+
	5	+	–
	6	–	–
	7	–	–
	8	+	+
	9	+	++
	10	++	++
	11	+	–
	12	+	+
	13	+	–
	14	+	–
	15	++	++
	16	+	–
	17	+	+
CHS2	**Plant no.**	**White Light**	**UV Light**
	1	–	–
	2	+++	+++
	3	++	–
	4	++	–
	5	++	–
	6	+	+
	7	++	+
	8	++	++
	9	+	–
	10	+	+
	11	+	++
	12	+	–
	13	+	+
	14	–	–
	15	+	–
	16	+	+
	17	+	+
	18	+	–
	19	+	–
	20	+	–

CHS3	Plant no.	White Light	UV Light
	1	++	+
	2	++	+
	3	++	+
	4	++	++
	5	++	++
	6	++	++
	7	–	–
	8	++	+
	9	+	–
	10	++	++
	11	+	+
	12	+	+
	13	++	++
	14	+	+
	15	+	–
	16	++	++
	17	++ / +++	+++
	18	+	+
	19	+	+
	20	++ / +++	+++
	21	++ / +++	+++
	22	++ / +++	+++
	23	+	–
	24	+	–
	25	+	–
	26	+	+
	27	+	+
	28	++ / +++	+++
	29	+	+
	30	+	+
	31	++	++
	32	++	++
	33	++	++
	34	+	+
	35	+	+
	36	+	+

silencing constructs and is expected to be linked to the *CHS* silencing construct after integration onto the plant genome. Therefore, the Kanamycin resistance trait is indicative of the presence of a *CHS* transcriptional silencing construct. For each of the tested plant lines, most of the T2 seedlings were resistant to Kanamycin and none displayed 3:1 segregation, indicating that all the chosen plant lines had multiple transgene copies (Table 4.5). This finding was also confirmed by Southern blotting (data not shown).

The level of *CHS* mRNA in plants carrying constructs for transcriptional silencing of the *CHS* gene was investigated using Northern Blotting and Reverse Transcriptase Polymerase Chain Reaction (RT-PCR). Both Northern Blotting and RT-PCR indicated that plant lines CHS1/3, CHS1/10, CHS2/8, CHS3/17, CHS3/20 and CHS3/21 did not produce detectable amounts of *CHS* mRNA (Fig 4.15 and Fig 4.16). However, both methods detected a small amount of *CHS* mRNA in plant lines CHS1/15, CHS2/2 and CHS2/11 (Fig 4.15 and Fig 4.16). This finding was somewhat surprising since the phenotype of plant line CHS2/2 was indicative of very good CHS silencing.

As antibodies to the CHS protein are not commercially available, I tested for the presence of CHS activity by detecting the products of its catalysis in *A. thaliana* seed. Chalcone synthase catalyses the fourth step in the anthocyanin synthesis pathway and its downstream products include flavanones and anthocyanins. To test for the presence of these compounds, flavanones and anthocyanins were extracted from seed of the 9 selected plant lines, the CHS mutant (*tt4*) and wild type *A. thaliana* seed. The extracts were analysed using thin layer chromatography (TLC) (Fig. 4.17). TLC analysis revealed that most of *A. thalaiana* plant lines carrying CHS transcriptional silencing

Table 4.5: Segregation observed in T2 seed carrying constructs for transcriptional silencing of the *CHS* gene. T2 seed from the plant lines displaying Chalcone Synthase silencing were germinated on a medium containing Kanamycin. Resistance to Kanamycin was evaluated 3 weeks post germination.

Plant Line	Plate	Total number of seeds	Not germinated	Kanamycin resistant (Kan R)	Kanamycin sensitive (Kan S)	Kan R/ Kan S
CHS1/3	1	60	5	52	3	17:1
	2	60	6	52	2	26:1
CHS1/10	1	60	4	50	6	8:1
	2	60	8	48	4	12:1
CHS1/15	1	60	2	53	5	11:1
	2	60	7	50	3	17:1
CHS2/2	1	60	6	53	1	53:1
	2	60	10	50	0	50:0
CHS2/8	1	60	9	49	2	24:1
	2	60	8	50	2	25:1
CHS2/11	1	60	6	52	2	26:1
	2	60	6	51	3	17:1
CHS3/17	1	60	10	48	2	24:1
	2	60	8	50	2	25:1
CHS3/20	1	60	12	45	3	15:1
	2	60	5	51	4	13:1
CHS3/21	1	60	7	51	2	25:1
	2	60	9	48	3	16:1

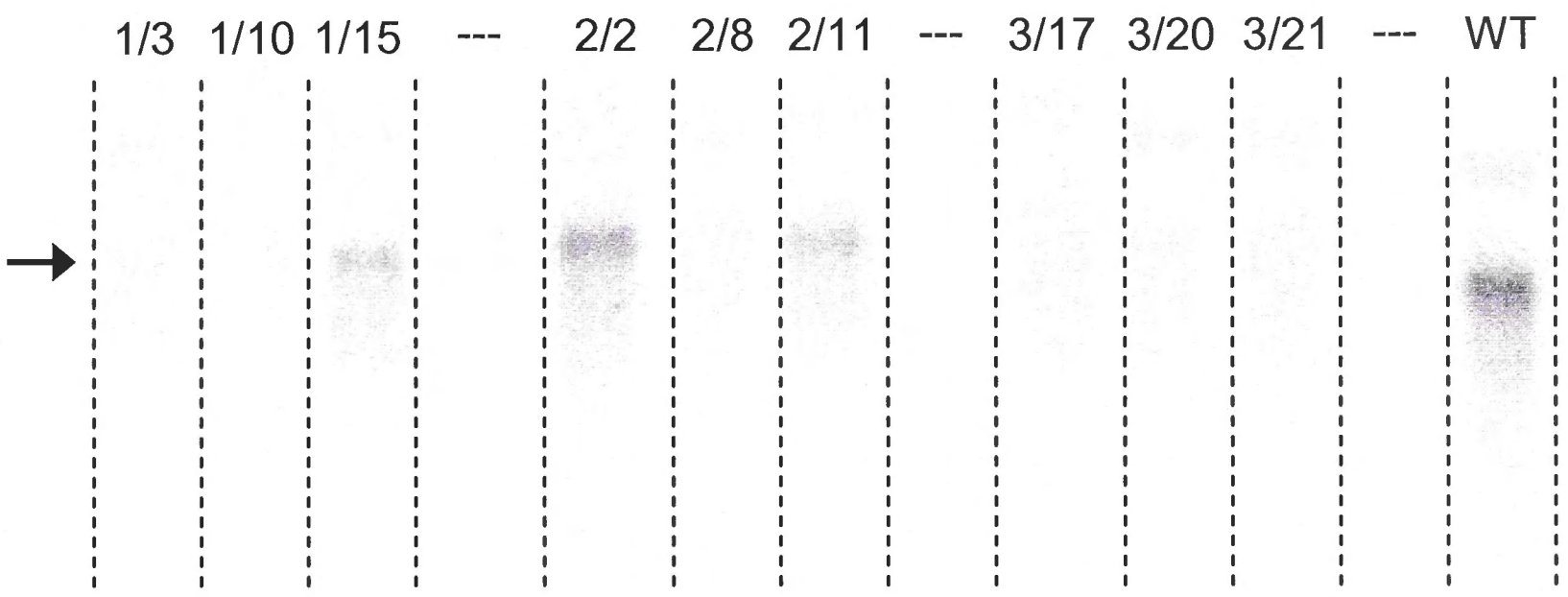


Fig. 4.15: Northern blot analysis of *CHS* mRNA levels in plants carrying CHS hpRNA constructs.

Seed from plant lines 1/3, 1/10, 1/15, 2/2, 2/8, 2/11, 3/17, 3/20 and 3/21 was germinated on MS medium containing Kanamycin. WT seed was germinated at the same time, but on MS medium that did not have Kanamycin selection.

Within two weeks after the germination leaf tissue was collected from at least five plants from each of the analysed plant lines.

RNA was extracted, analysed by Northern blotting and imaged as described in section 4.2.6.

The identity of each sample is indicated above the appropriate lane. Lanes that do not contain a sample are denoted with '---'. The arrow indicates the expected size of *CHS* transcript (1.35kb)

Fig. 4.16: Analysis of *CHS* RNA levels using Reverse Transcriptase - Polymerase Chain Reaction (RT-PCR).

Seed from plant lines 1/3, 1/10, 1/15, 2/2, 2/8, 2/11, 3/17, 3/20 and 3/21 was germinated on MS medium containing Kanamycin. WT seed was germinated at the same time, but on MS medium that did not contain Kanamycin selection.

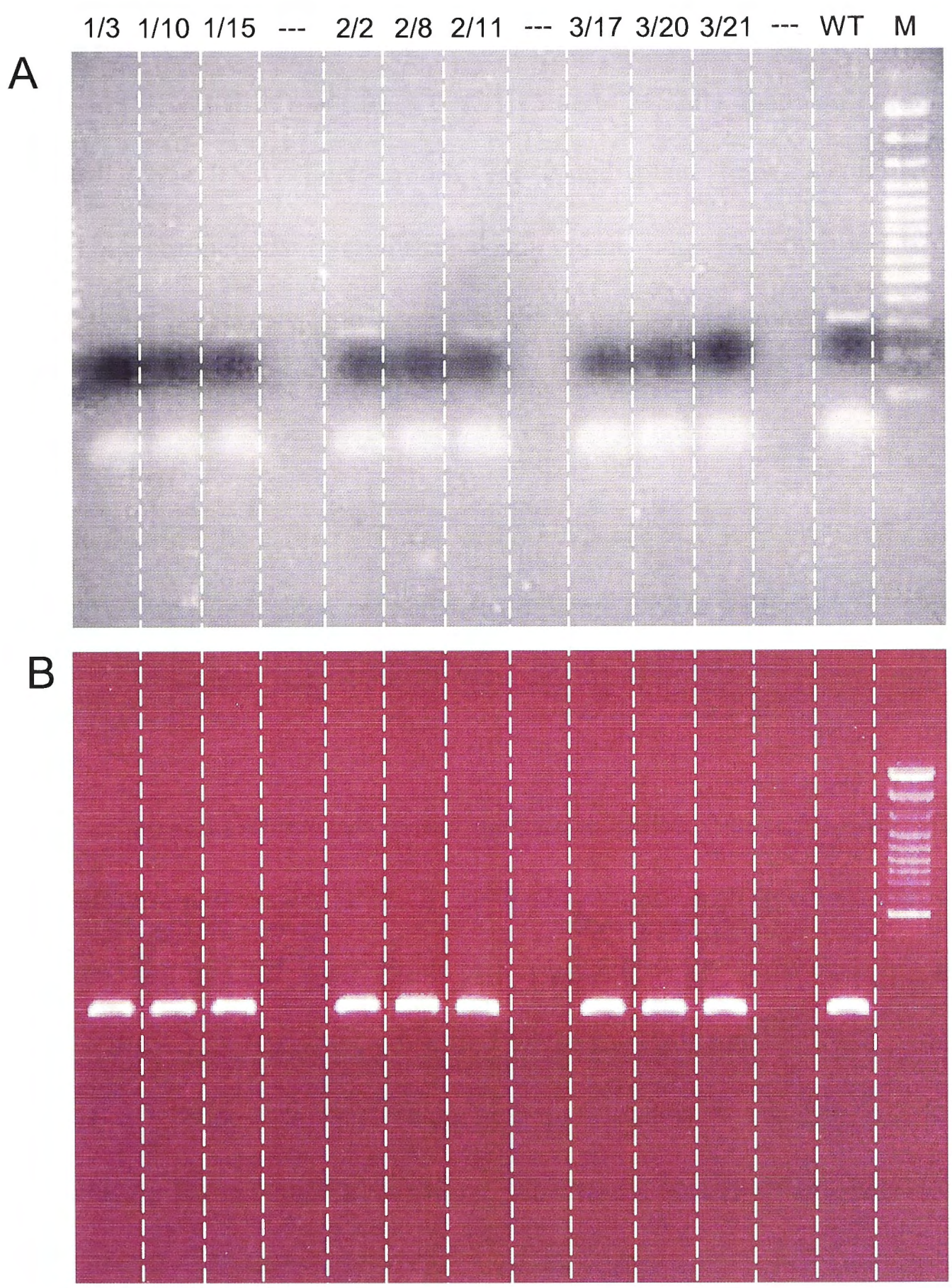
Within two weeks after the germination leaf tissue was collected from at least five plants from each of the analysed plant lines.

RNA was extracted and analysed by RT-PCR as described in section 4.2.7.

The identity of each sample is indicated above the appropriate lane. Lanes that do not contain a sample are denoted with '---'. The lanes containing the Gene Ruler DNA Ladder 100bp+ markers (Fermentas, Lithuania) are denoted with M.

A: Amplification using primers specific to the *CHS* gene.
Samples were taken after 24 PCR cycles.

B: Amplification using primers specific to 18S RNA
Samples were taken after 24 PCR cycles.


1/3
1/10
1/15

2/2
2/8
2/11

3/17
3/20
3/21

WT
M
A
B

constructs did not accumulate detectable levels of flavanone or anthocyanin compounds. Trace amounts of these compounds were detected in plant lines CHS1/10, CHS2/2, CHS2/8 and CHS3/17. The presence of the trace amount of flavanone and anthocyanin compounds in the CHS2/2 sample was consistent with the presence of small amounts of CHS RNA in this plant line. However, flavanone and anthocyanin compounds were not detected in plant lines CHS1/15 and CHS2/11 that also produce small amounts of CHS RNA. Furthermore, all tested plant lines accumulated fluorescent aromatic compounds (cinnamate, 4-coumarate and 4-coumaroyl-CoA), which are anthocyanin precursors, and appear in the anthocyanin synthesis pathway upstream from the step catalysed by chalcone synthase. When the TLC plates were observed under UV light, these compounds appeared as light-blue fluorescent bands (Fig. 4.17 – marked with blue arrows). These compounds are also responsible for the fluorescence observed in whole seed collected from plants with a silenced CHS gene (Fig. 4.14).

The levels of flavanone and anthocyanin compounds were further evaluated using high performance liquid chromatography (HPLC). All 9 plant lines carrying constructs for transcriptional silencing of CHS were found to have significantly reduced levels of these compounds (Fig 4.18). However, compared to the *tt4* CHS mutant, these plants had higher levels of compounds that eluted at approximately 4 minutes and 5.2 minutes. Unfortunately these compounds did not correspond to any of the standards and could not be identified in this experiment.

Figure 4.17: Thin Layer Chromatography (TLC) analysis of flavanone and anthocyanin compounds extracted from seeds harvested from *A. thaliana* plant lines carrying CHS1, CHS2 or CHS3 hpRNA constructs.

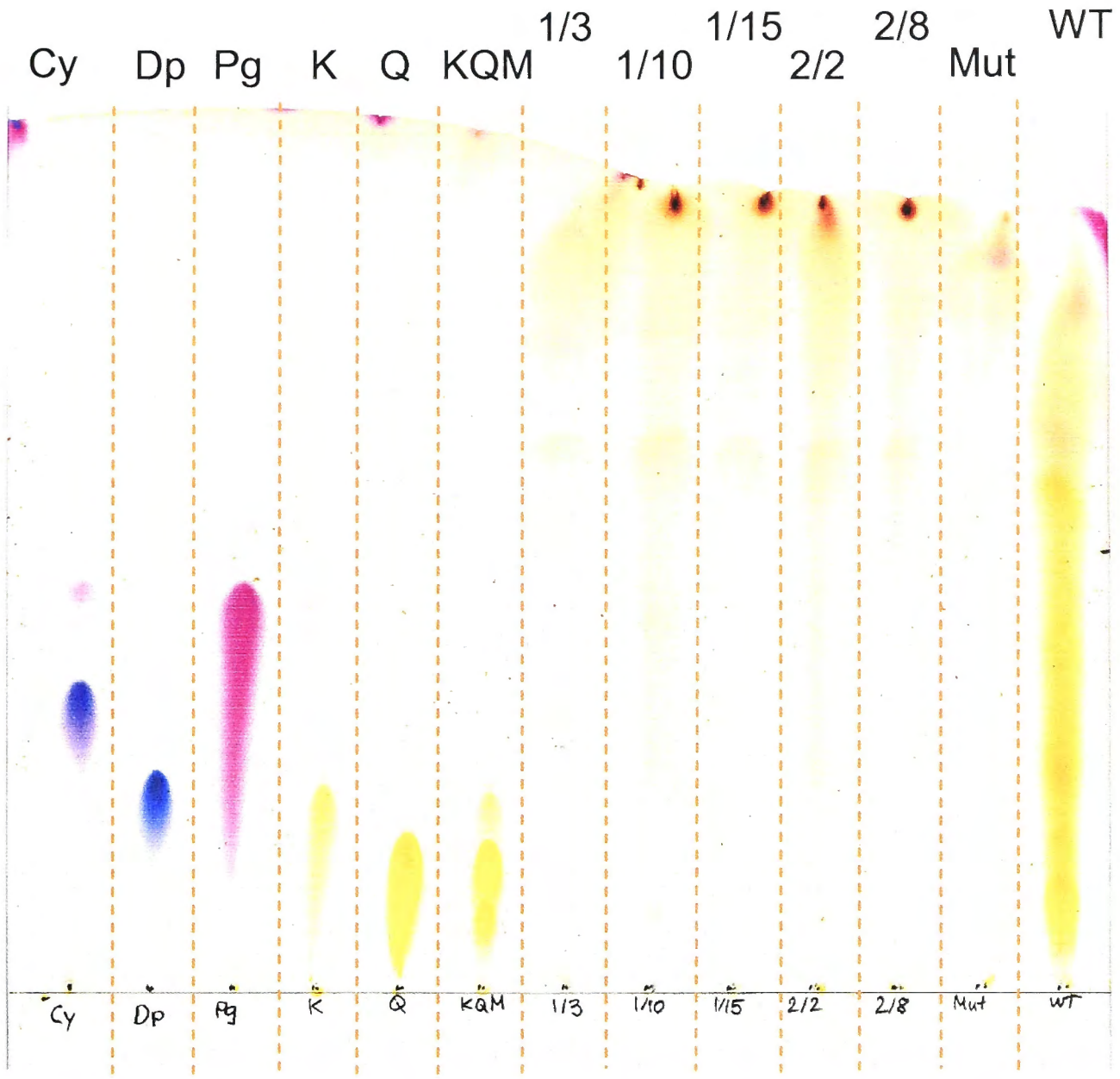
Anthocyanin compounds were extracted from seeds collected from at least 5 individual plants from each of the tested plant lines. The extract was analysed as described in section 4.2.8. The identity of the plant line from which the extract was obtained is marked above each lane.

A: A TLC plate observed under white light.

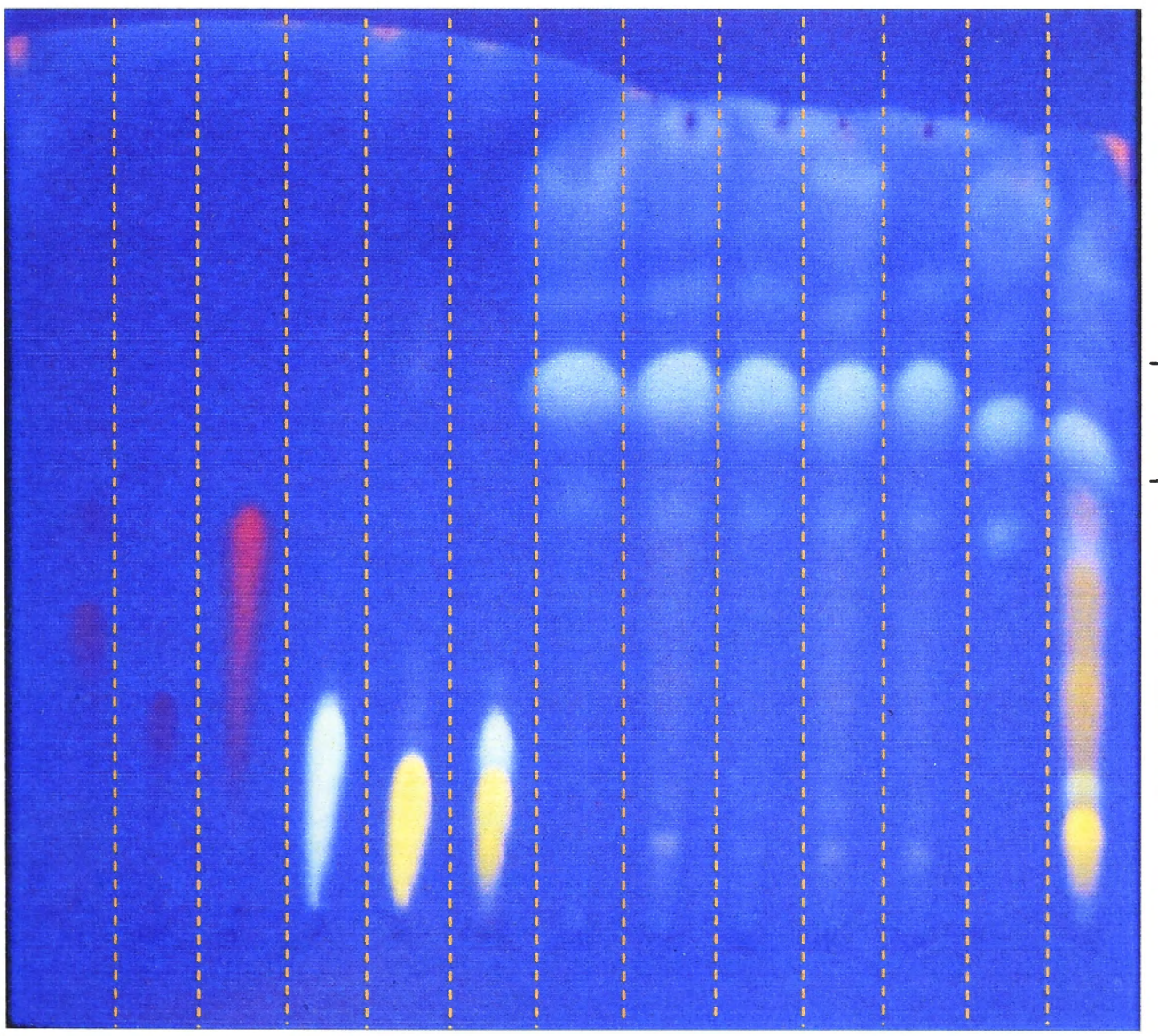
B: The same TLC plate observed under long wave UV light. The blue arrow marks the position of florescent aromatic compounds (cinnamate, 4-coumarate and 4-coumaroyl-CoA), which are anthocyanin precursors, that occur in the anthocyanin synthesis pathway upstream from the step catalysed by chalcone synthase.

Abbreviations:
Cy = cyanidin, Dp = delphinidin, Pg = pelargonidin, K = kaempferol, Q = quercetin, KQM = mixture of kaempferol and quercetin. For position of these compounds in the flavonoid synthesis pathway see Fig. 4.9.

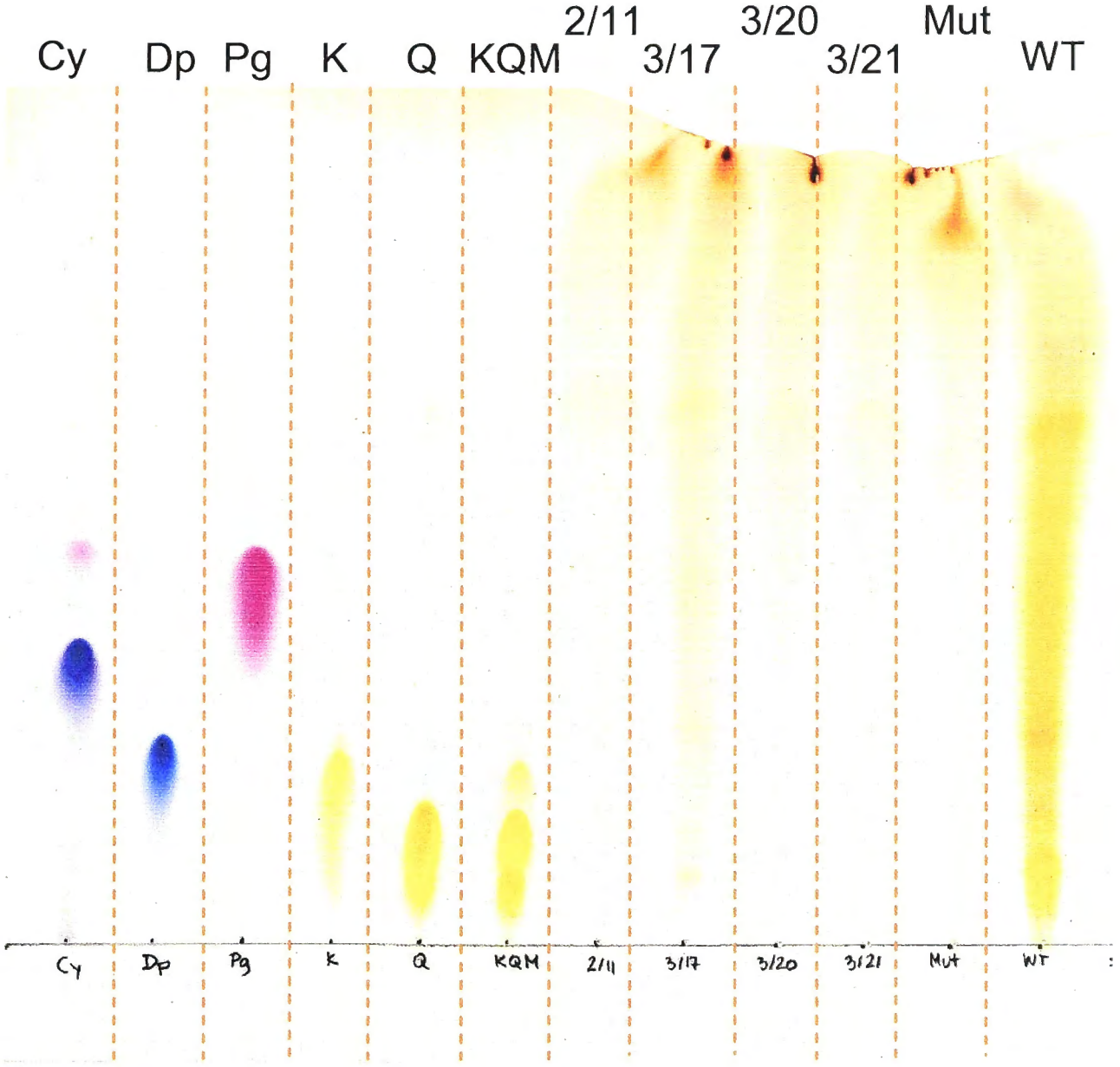
White light



UV light



White light



UV light

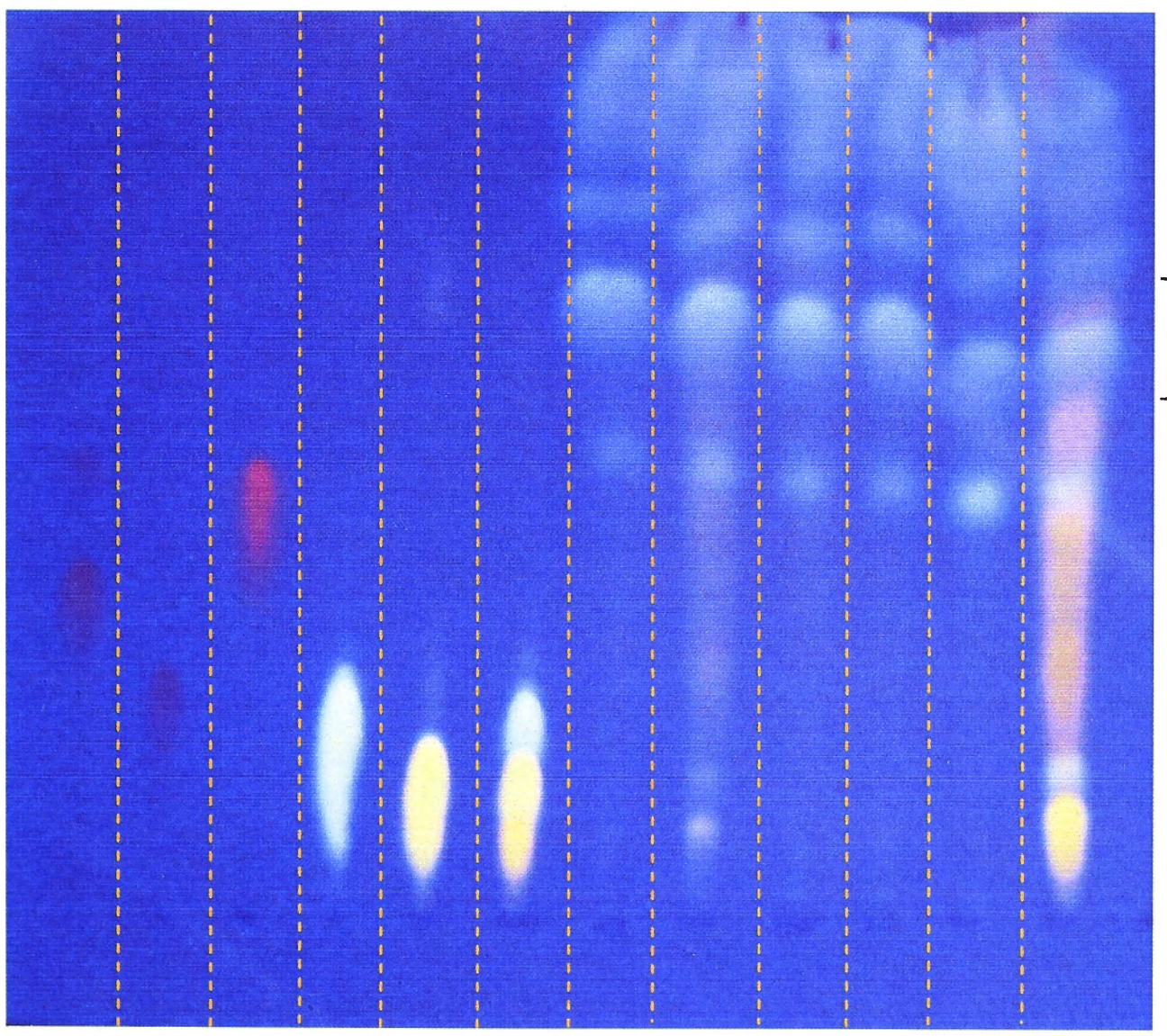


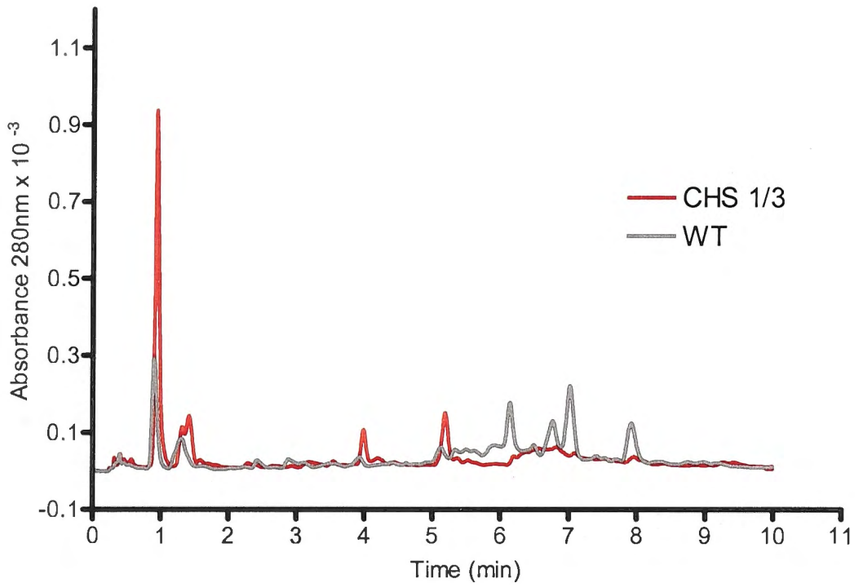
Figure 4.18: High Performance Liquid Chromatography (HPLC) analysis of flavanone and anthocyanin compounds extracted from *A. thaliana* plant lines carrying CHS1, CHS2 or CHS3 hpRNA constructs.

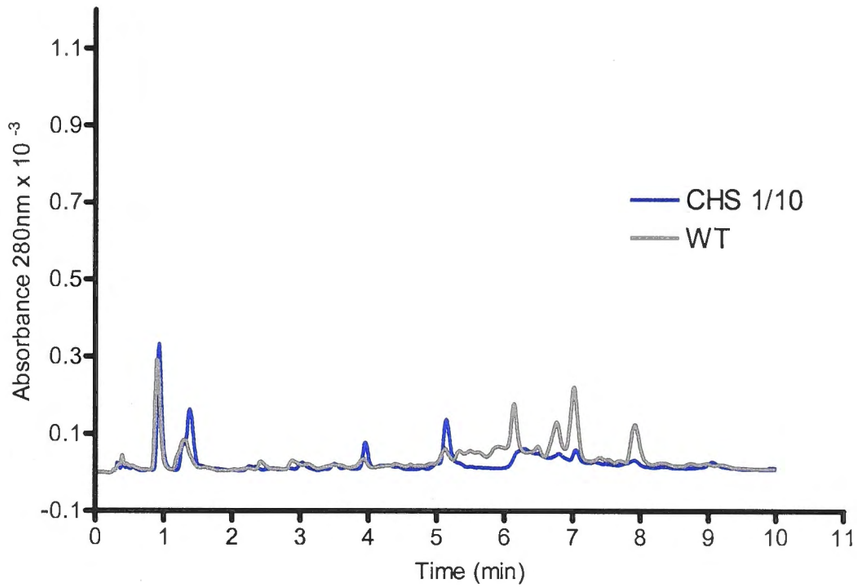
Anthocyanin compounds were extracted from seed collected from at least 5 individual plants from each of the tested plant lines. The extract was analysed as described in section 4.2.8.

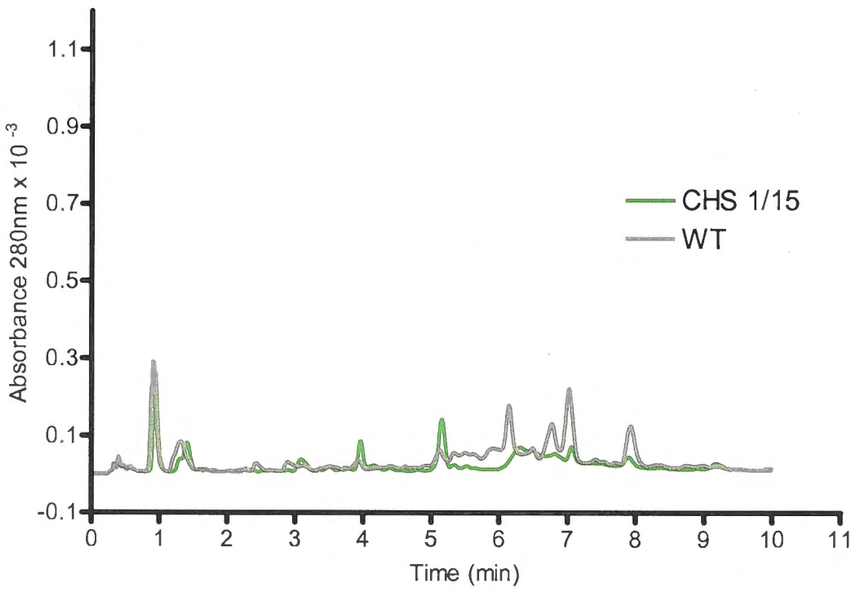
A: Analysis of compounds extracted from plants lines carrying CHS1 construct
B: Analysis of compounds extracted from plants lines carrying CHS2 construct
C: Analysis of compounds extracted from plants lines carrying CHS3 construct

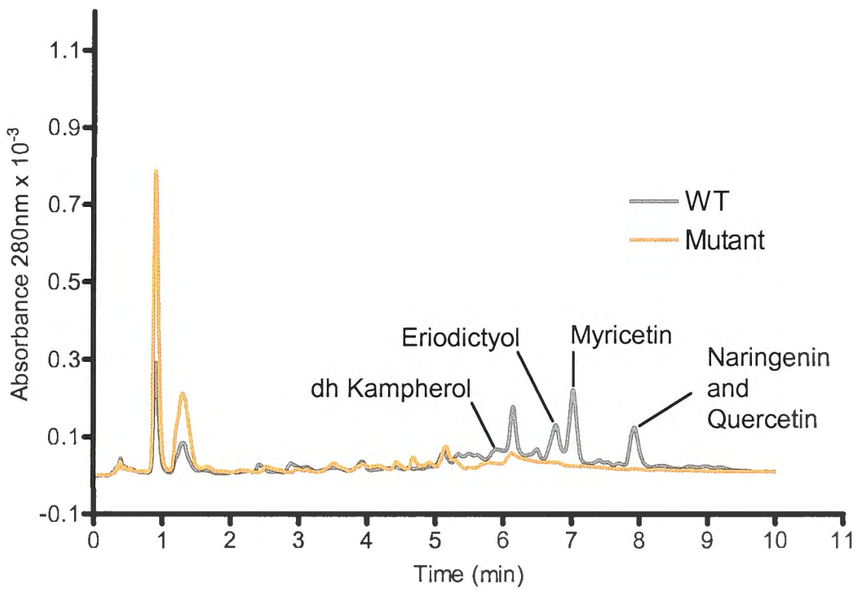
On each of the panels the graph located in the bottom right hand corner depicts comparison of HPLC profiles obtained by analysing seed harvested from a WT plant and CHS mutant (*tt4*) plant. Major peaks in HPLC profiles are labeled with identities of compounds responsible for these peaks.

A: CHS1 Plant lines

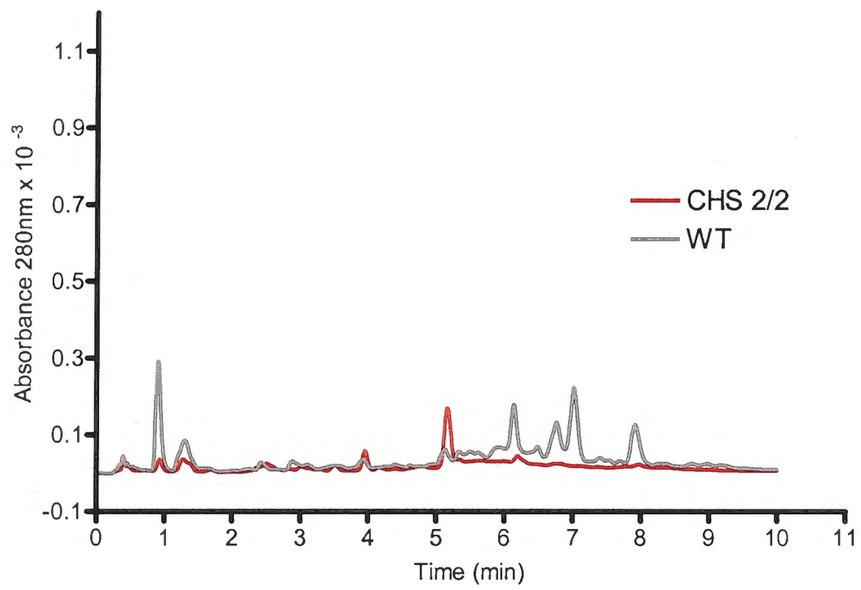


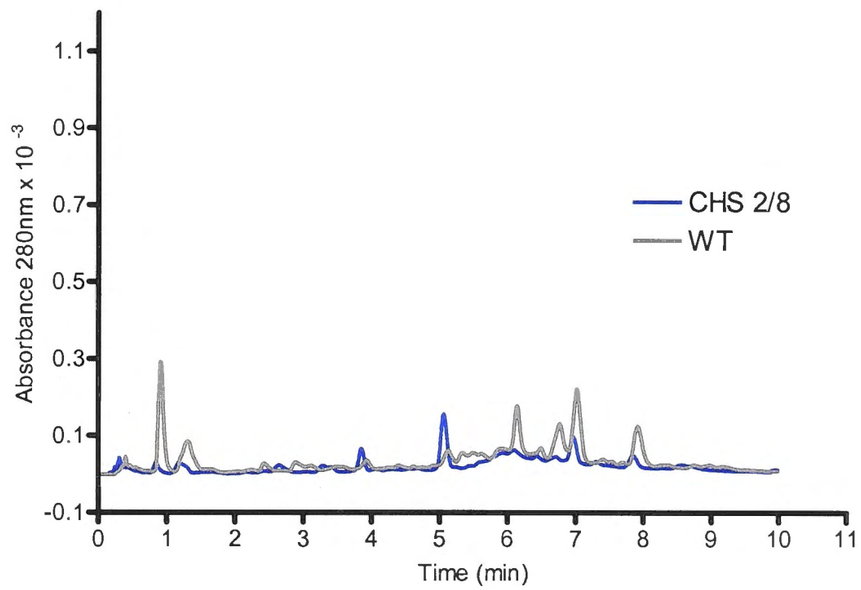


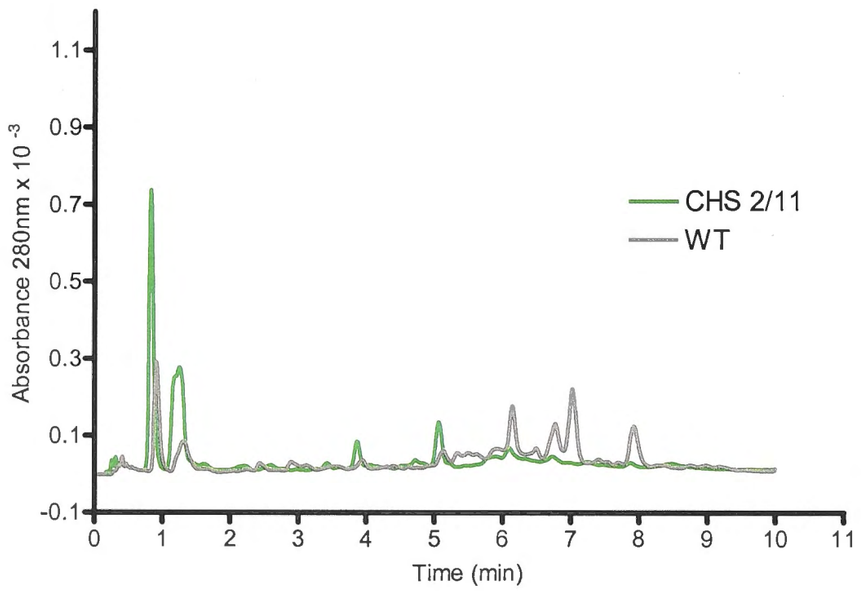


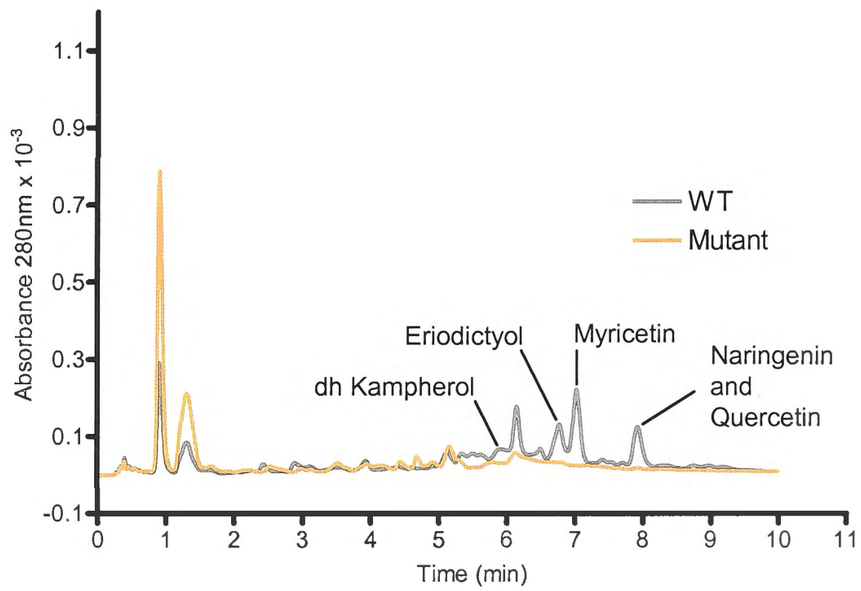


B: CHS2 Plant lines

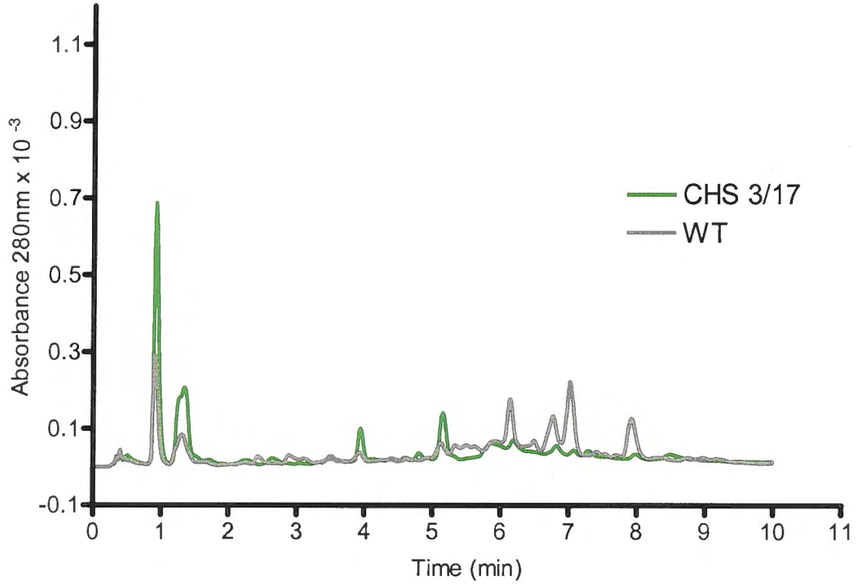


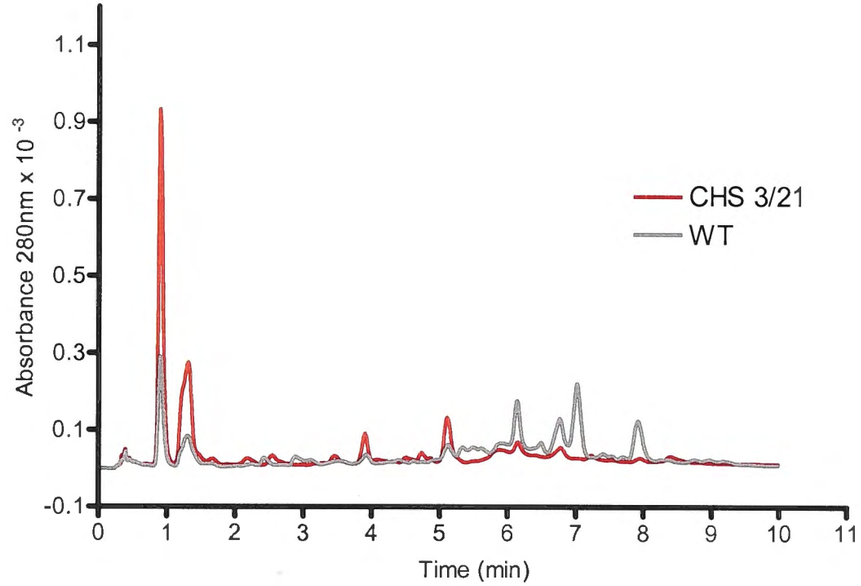


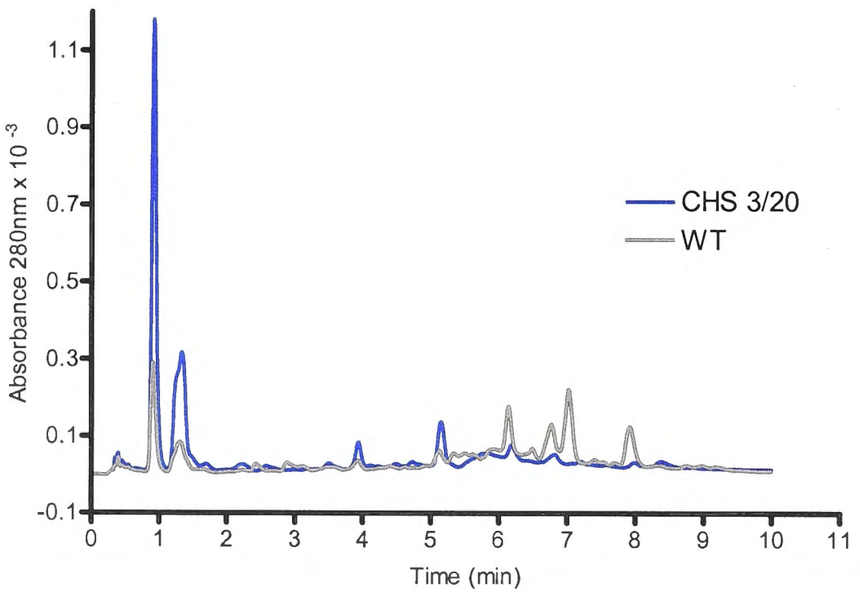


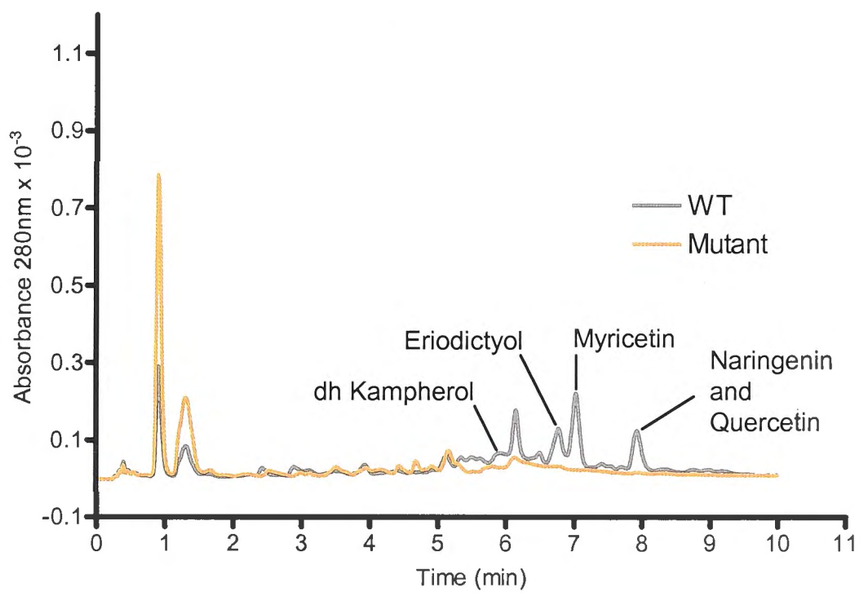


C: CHS3 Plant lines









4.3.2 DNA methylation patterns associated with TGS of CHS

The level of methylation was determined by calculating the percentage of methylated cytosine residues out of the total number of analysed cytosine residues. A sample calculation is shown in Table 4.6.

The primers used to amplify bisulfite converted DNA were designed as outlined by Clark *et al.* (1994). To achieve successful amplification and sequencing, the size of the PCR products was limited to no more than 500bp in length. Primer positions and expected amplification products are shown in Fig. 4.19.

Depending on the plant line being analysed and the primer pair being used, the bisulfite PCR products could be amplified either from both the endogenous gene and the transgene or from the endogenous gene only (Fig 4.19). This allowed the methylation levels present in the endogenous gene to be compared with those present in the transgene.

4.3.2.1 Levels of transgene and endogenous gene methylation

Using the various primer pairs, amplification from bisulfite treated DNA extracted from plant lines carrying the CHS1 hairpin constructs generated four different PCR products: BisCHS1, BisCHS2, BisCHS3 and BisCHS4 (Fig. 4.20). BisCHS1 and BisCHS2 PCR products were obtained using primer sets that had at least one primer complementary to the sequences present in the endogenous gene, but not in the transgene. Consequently, BisCHS1 and BisCHS2 could only be amplified when the endogenous *CHS* gene was used as a template (Fig. 4.20). In contrast, BisCHS3 and BisCHS4 Bisulfite PCR products were generated using primer sets that annealed to

Table 4.6: A sample of calculations used to determine percentage of methylation.

Plant Line		CHS 2/2	CHS 2/8	CHS 2/11	WT
No. of sequences		24	22	11	21
Type	Position	No. of methylated C residues			
CG	-152	20	20	11	0
CG	-98	10	18	11	0
CG	-80	11	16	1	0
CG	-42	6	9	10	0
CG	-10	2	4	0	0
Total no.of met. Cs = sum of met Cs (values given in the columns)		49	67	33	0
Total no. of C residues = no. of Cs being analysed (5) **X** no. of sequences		120	110	55	105
% Methylation = Total no. of Cs / Total no. of methylated Cs		**40.83**	**60.91**	**60.00**	**0.00**

Figure 4.19: Schematic representation of the *CHS* promoter, hpRNA transgenes and the expected amplification products from the bisulfite PCR.

The pink bar represents the endogenous *CHS* promoter. The orange section of this bar represents the coding region of the endogenous *CHS* gene.

The blue lines with arrows mark regions of *CHS* promoter amplified by specific sets of primers. Each amplification product was named according to the set of primers used for the amplification.

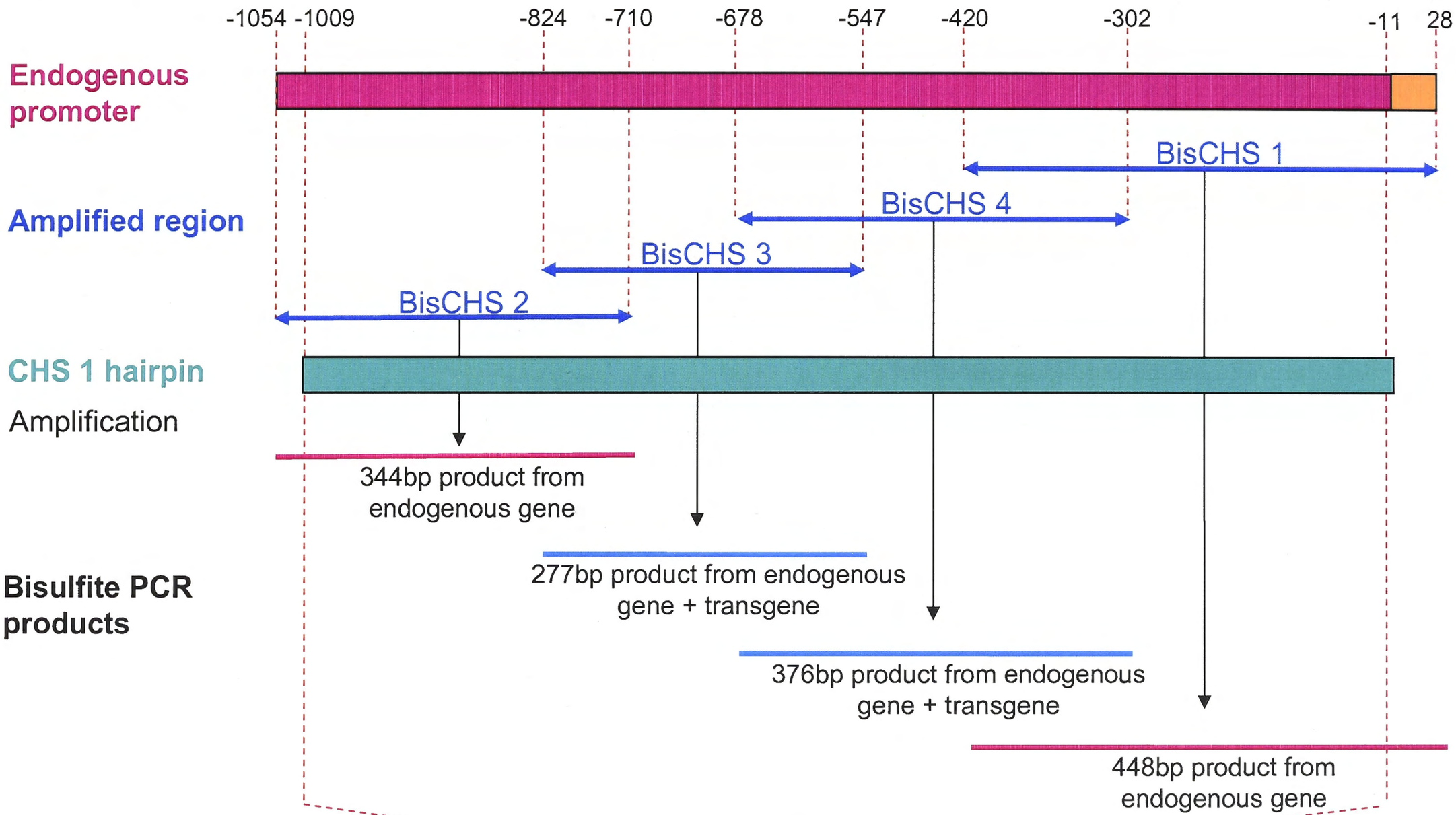
Green, black and yellow bars represent CHS1, CHS2 and CHS3 hpRNA transgenes, respectively.

A: The products obtained by amplification from DNA extracted from plant lines carrying the CHS1 construct. The pink lines represent products generated by amplification from the endogenous gene only. Blue lines represent amplification products that contain a mixture of molecules - some amplified from the endogenous gene and some from the transgene.

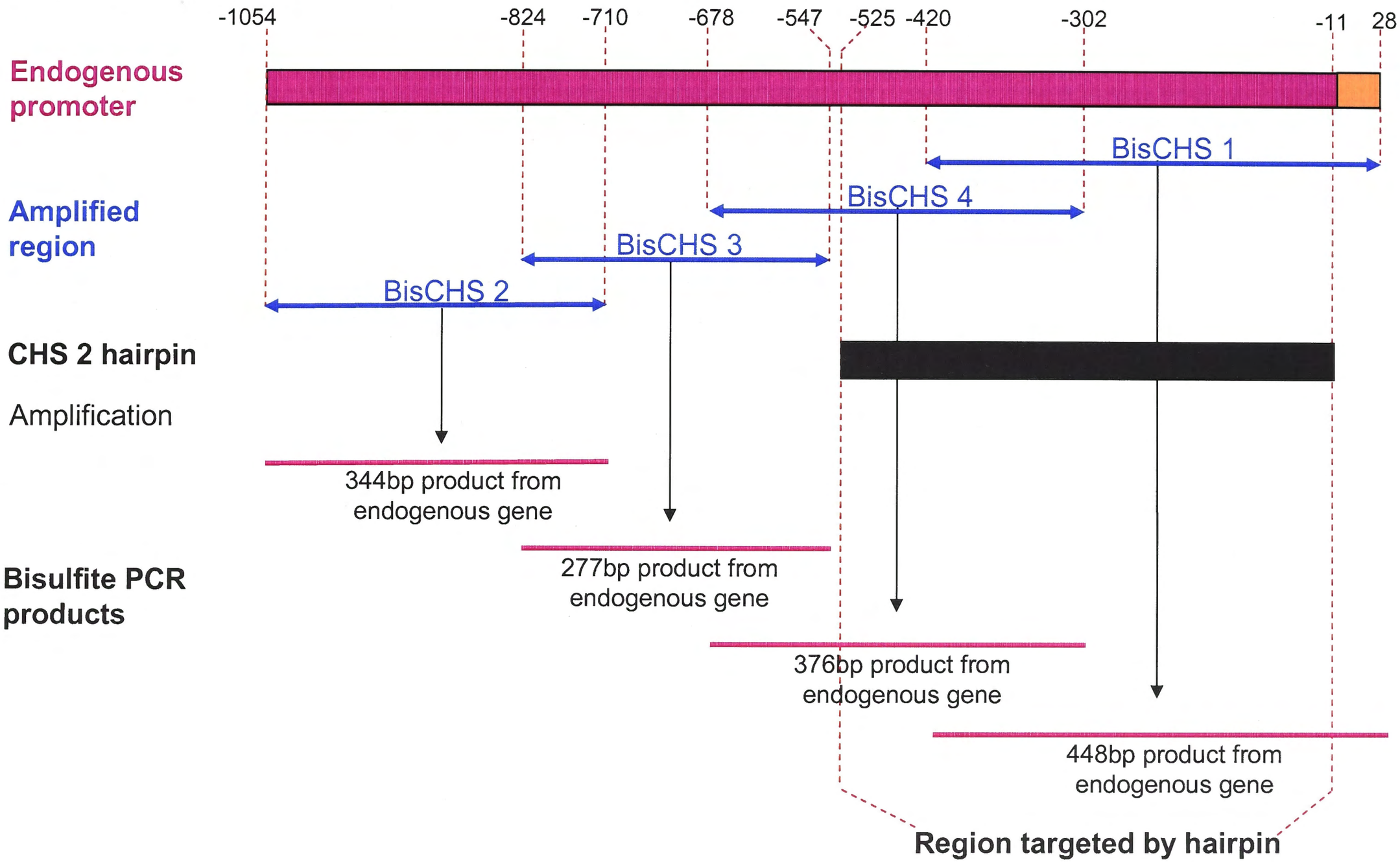
B: The products obtained by amplification from DNA extracted from plant lines carrying the CHS2 construct.

C: The products obtained by amplification from DNA extracted from plant lines carrying the CHS3 construct.

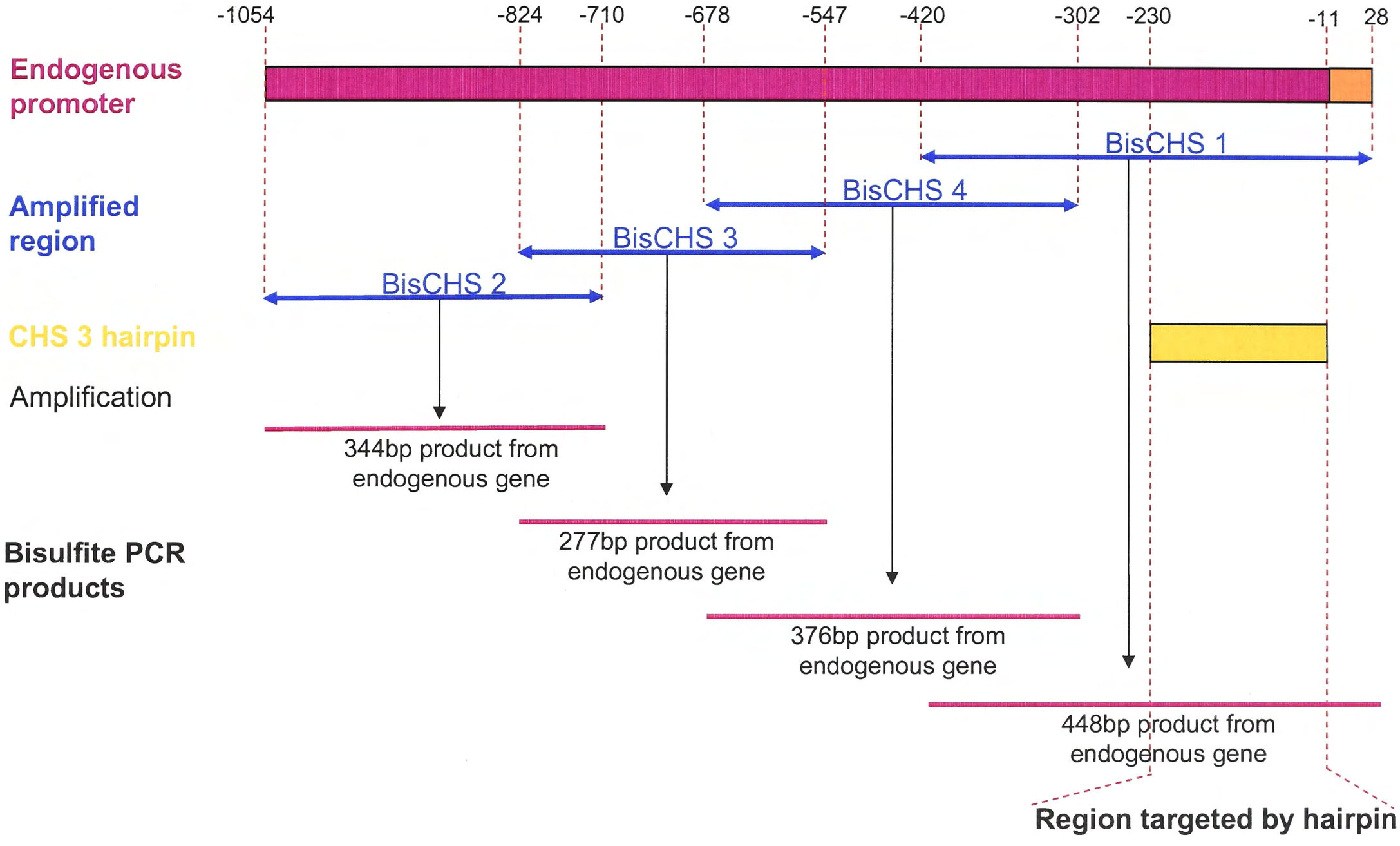
A: CHS1 Plant lines



B: CHS2 Plant lines



C: CHS3 Plant lines



sequences present both in the transgene and the endogenous *CHS* gene. Consequently, BisCHS3 and BisCHS4 products contained a mixture of molecules – some of these molecules were generated by amplification from the transgene, while others were generated by amplification from the endogenous *CHS* gene (Fig. 4.20).

Two sections of the *CHS* promoter were amplified by more than one set of primers. For example, both BisCHS1 and BisCHS4 products contained sequence located between -420 and -302. Similarly, BisCHS2 and BisCHS3 products both contained sequence located between -824 and -710. The fact that methylation of the same sequence could be analysed using different bisulfite PCR products was used to indirectly compare methylation levels present in the endogenous gene with those present in the transgene. The levels of methylation of the endogenous *CHS* gene in the region located between -420 and -302 and between -824 and -710 were determined by sequencing BisCHS1 and BisCHS2 products respectively. The methylation levels of these regions were also analysed by sequencing BisCHS3 and BisCHS4 products. However, because BisCHS3 and BisCHS4 products contained molecules amplified from both the endogenous *CHS* gene and those amplified from the transgene, methylation levels obtained by this analysis were likely to reflect an average level of methylation for the endogenous gene and the transgene.

A comparison of the methylation data obtained by sequencing BisCHS1 and BisCHS2 products (amplification from the endogenous gene only) and BisCHS3 and BisCHS4 products (amplification from both the endogenous gene and the transgene) suggests that the endogenous gene and the transgene had very similar level of symmetric

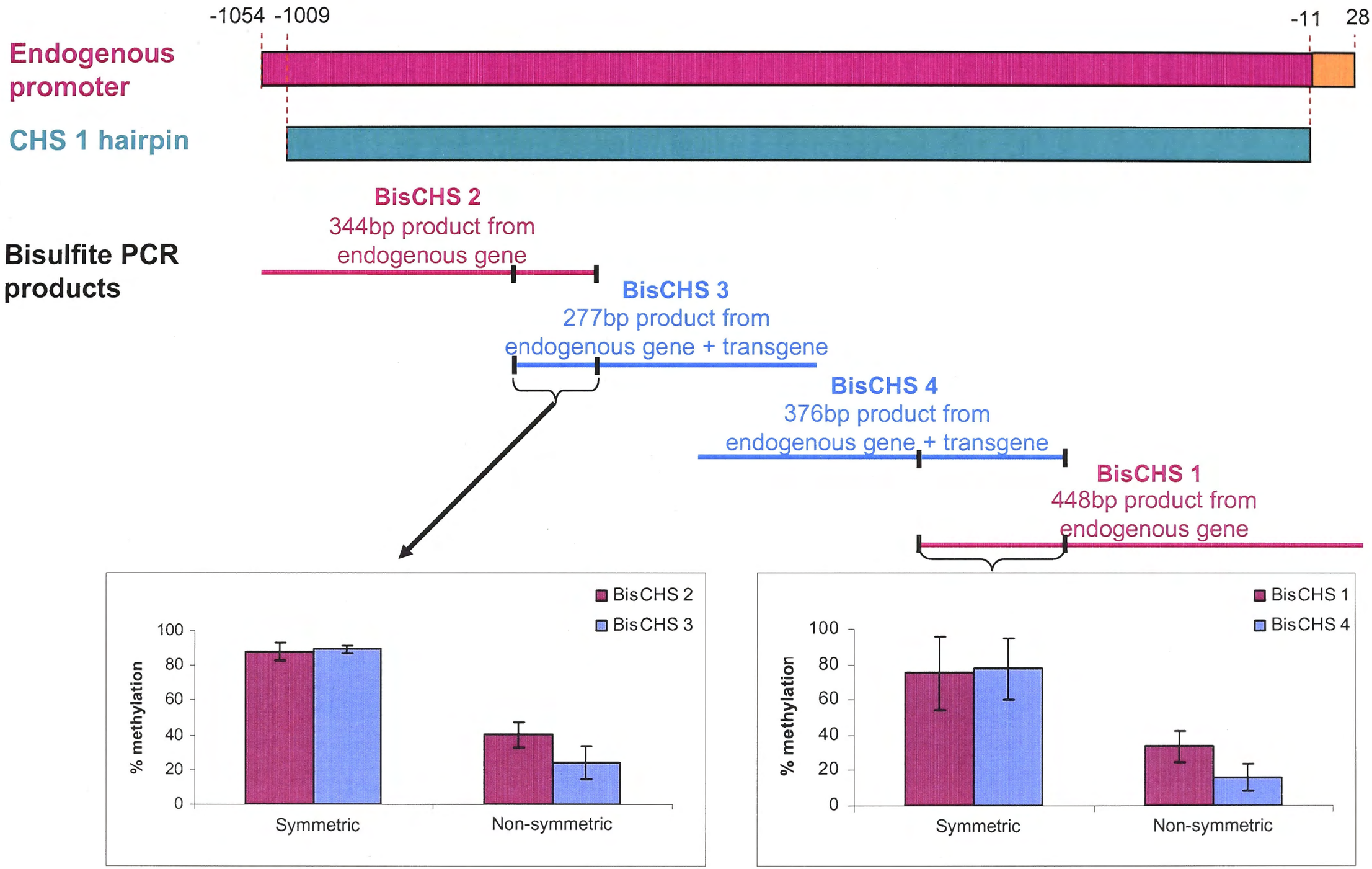
Fig. 4.20: Indirect comparison of methylation levels observed in transgene and the endogenous gene.

The pink bar represents the endogenous *CHS* promoter. The green bar represents CHS1 hpRNA transgene. CHS1 hpRNA transgene was derived from the -11 to -1009 segment of the endogenous *CHS* promoter.

Pink lines represent amplification products generated by amplification from the endogenous gene only (BisCHS1 and BisCHS2). Blue lines represent amplification products that contain a mixture of molecules - some amplified from the endogenous gene and some from the transgene (BisCHS3 and BisCHS4).

Brackets mark the segments of the *CHS* promoter that were amplified by more than one set of primers. The level of methylation of endogenous *CHS* gene in the bracketed region (from -420 to -302 and from -824 to -710) was determined by sequencing BisCHS1 and BisCHS2 products respectively. The methylation level of these regions was also analysed by sequencing BisCHS4 and BisCHS3 products which contained both molecules amplified from the endogenous *CHS* gene and those amplified off the CHS1 hpRNA transgene.

The bar graphs represent the mean level of symmetric (CG and CnG) and non-symmetric cytosine methylation calculated by an analysis of the methylation levels in three plant lines carrying the CHS1 construct. Error bars represent standard deviation about the mean.


-1054
-1009
-11
28
Endogenous promoter
CHS 1 hairpin
Bisulfite PCR products
BisCHS 2
344bp product from endogenous gene
BisCHS 3
277bp product from endogenous gene + transgene
BisCHS 4
376bp product from endogenous gene + transgene
BisCHS 1
448bp product from endogenous gene
BisCHS 2
BisCHS 3
% methylation
100
80
60
40
20
0
Symmetric
Non-symmetric
BisCHS 1
BisCHS 4
% methylation
100
80
60
40
20
0
Symmetric
Non-symmetric

cytosine (CG and CnG) methylation while the endogenous gene had higher level of non-symmetric cytosine methylation. (Fig. 4.20).

The methylation levels measured by the BisCHS3 and BisCHS4 products can be assumed to reflect the average of endogene and transgene methylation. However, the plant lines analysed in these experiments contained more then one copy of the CHS1 transgene (Section 4.3.1), while there was only one copy of the endogenous *CHS* gene. Therefore, BisCHS3 and BisCHS4 products are likely to contain a higher proportion of molecules amplified from the transgene. As such, the methylation levels obtained from analysis of BisCHS3 and BisCHS4 are more likely to reflect methylation levels the transgene than the simple average of the transgene and endogene methylation levels.

4.3.2.2 Comparison of methylation levels in regions targeted by CHS Hp transgenes and regions that were not targeted by the CHS Hp transgenes

In an attempt to map the methylation patterns present in the *A. thaliana* plant lines carrying constructs for transcriptional silencing of the *CHS* gene, I analysed methylation levels in regions of the endogenous *CHS* gene that are homologous to the CHS Hp constructs and regions that did not share homology with the silencing constructs. As expected, regions of the endogenous *CHS* gene that were homologous to the silencing constructs were targeted by RNA directed DNA methylation and had a high percentage of methylated cytosine residues (Fig. 4.21, Fig. 4.22). In contrast, regions that had no homology with the silencing constructs, and were therefore not directly targeted by the transgene, had very low levels of methylation (Fig. 4.21, Fig. 4.22). In plant lines carrying the CHS1 silencing construct, levels of methylation in regions with no homology with CHS Hp construct were somewhat higher than those

Fig. 4.21: Comparison of methylation levels in sections of the endogenous *CHS* promoter targeted by the CHS hpRNA transgenes and sections that were not targeted by the CHS hpRNA transgenes.

Seed from plant lines 1/3, 1/10, 1/15, 2/2, 2/8, 2/11, 3/17, 3/20 and 3/21 was germinated on MS medium containing Kanamycin. WT seed was germinated at the same time, but on MS medium that did not contain Kanamycin.

Two weeks after germination, leaf tissue was collected from at least five plants from each of the analysed plant lines. DNA was extracted, treated with sodium bisulfite and the methylation status analysed, as described in section 4.2.11. The percentage of methylcytosines for each of the plant lines was calculated as outlined in table 4.5.

The pink bar represents the endogenous *CHS* promoter. Green, black and yellow bars represent CHS1, CHS2 and CHS3 hpRNA transgenes, respectively. Bars representing CHS hpRNA transgenes are positioned parallel to the section of the endogenous *CHS* promoter from which they were derived.

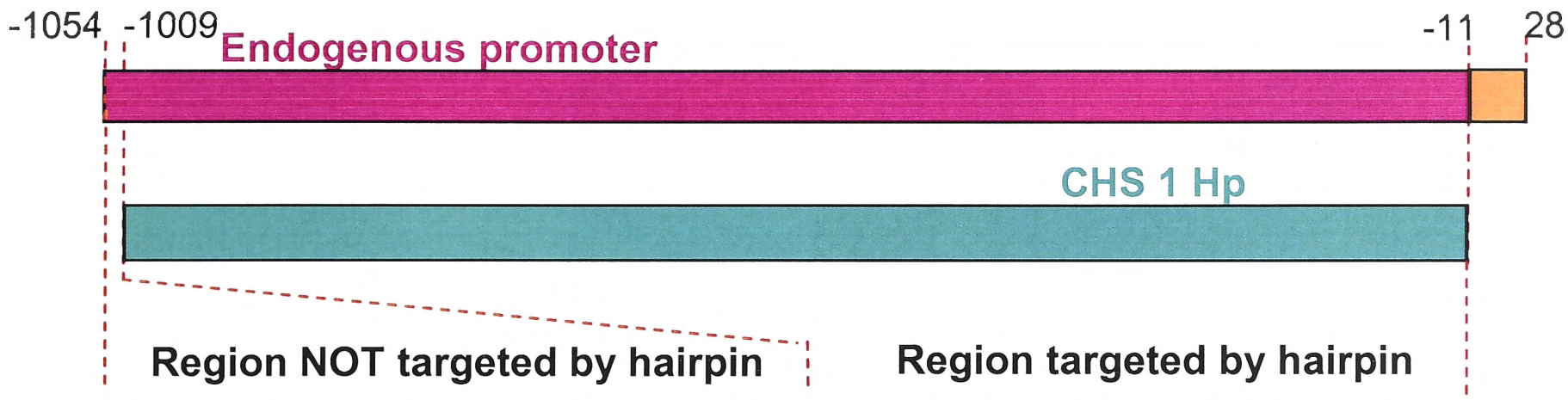
Methylation levels were calculated for three different types of cytosine residues:
I those adjacent to a guanine residue (CG);
II those separated from a guanine residue by another residue (CnG) and
III those that did not fall in one of the previous two categories (non-symmetric cytosine residues).

A: Methylation levels observed in plant lines carrying CHS1 hpRNA transgene

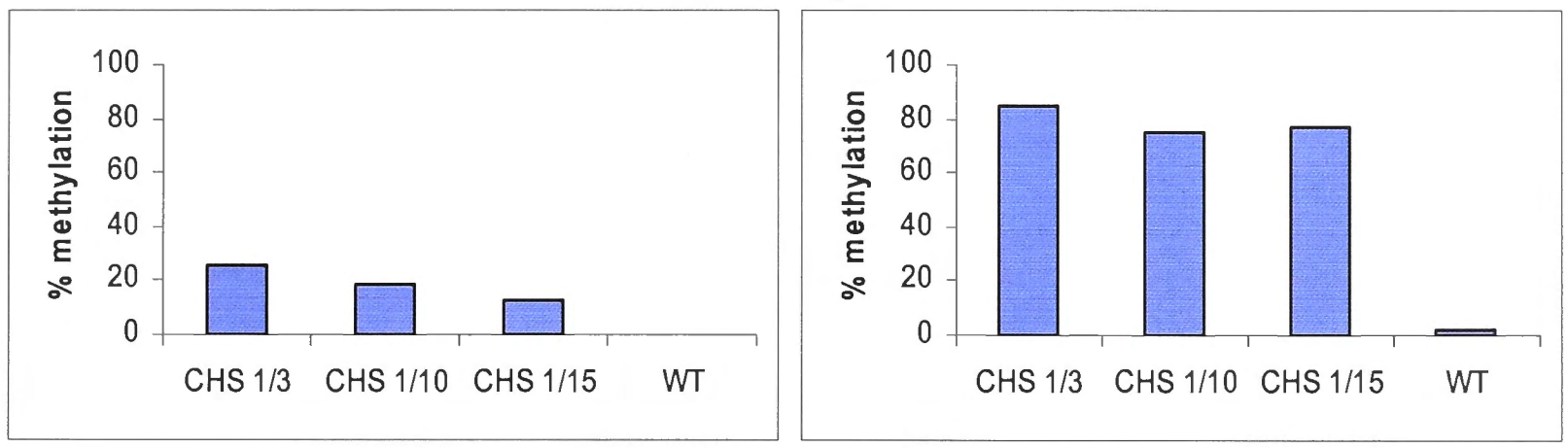
B: Methylation levels observed in plant lines carrying CHS2 hpRNA transgene

C: Methylation levels observed in plant lines carrying CHS3 hpRNA transgene

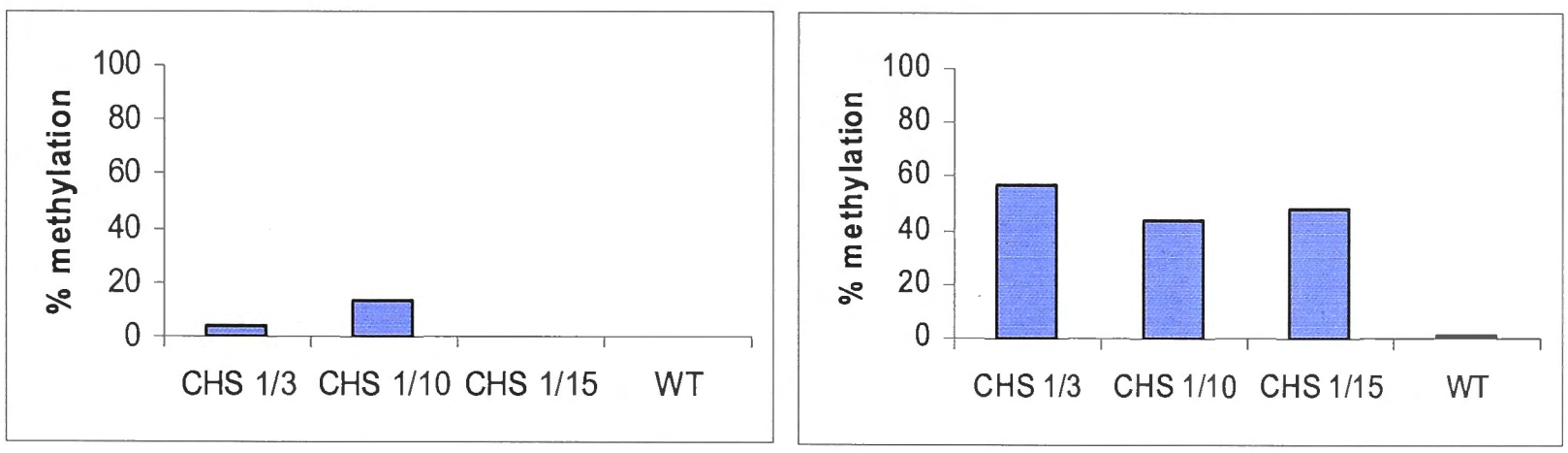
A: CHS1 Plant lines



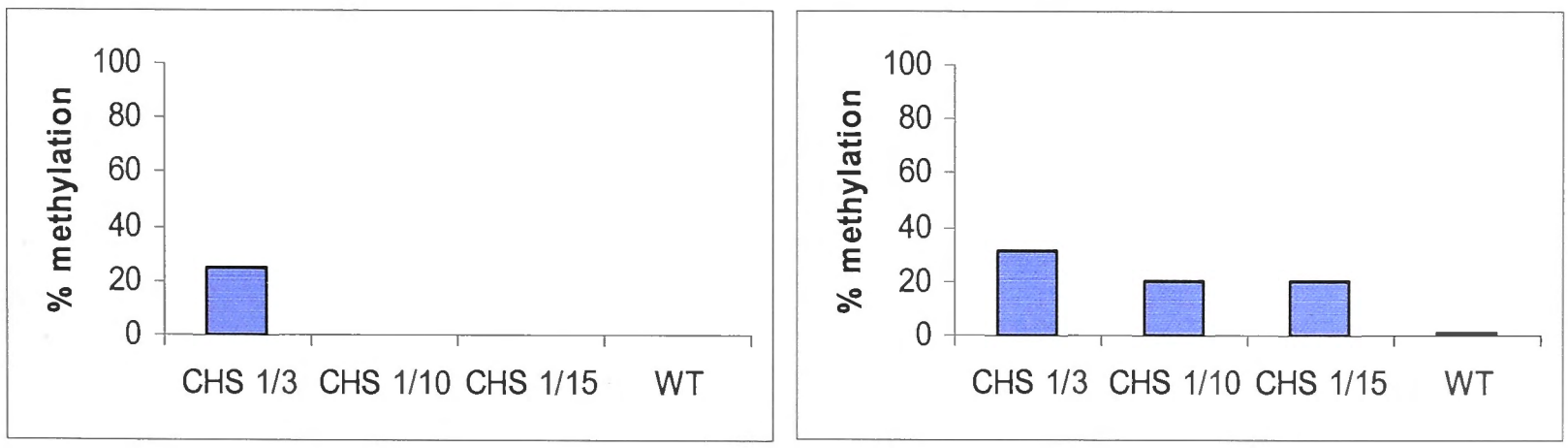
I CG methylation



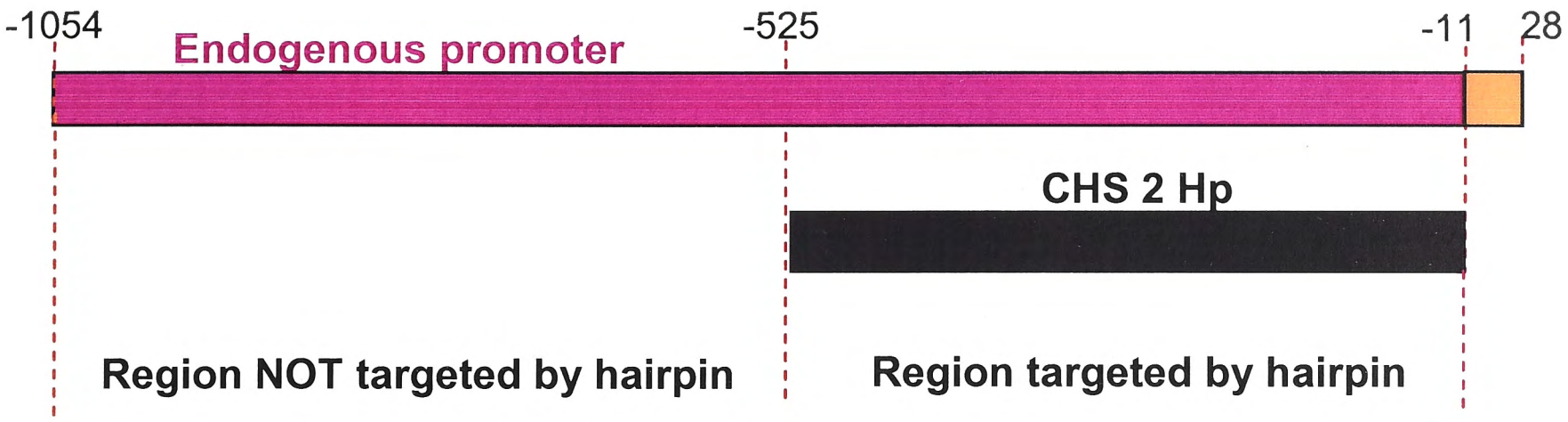
II CnG methylation



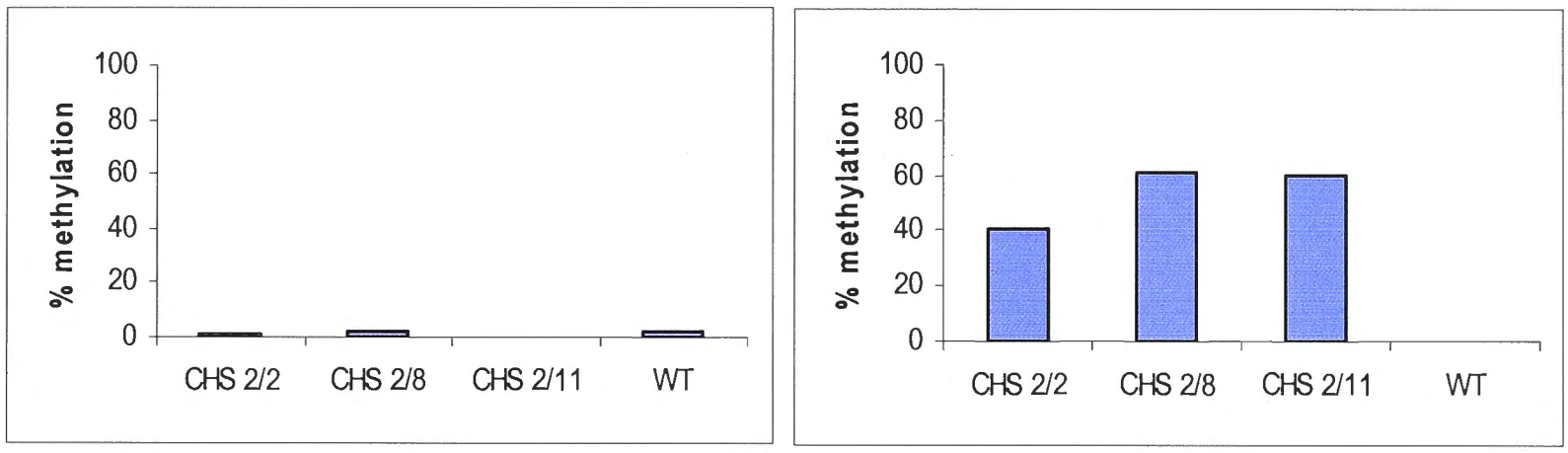
III non-symmetric methylation



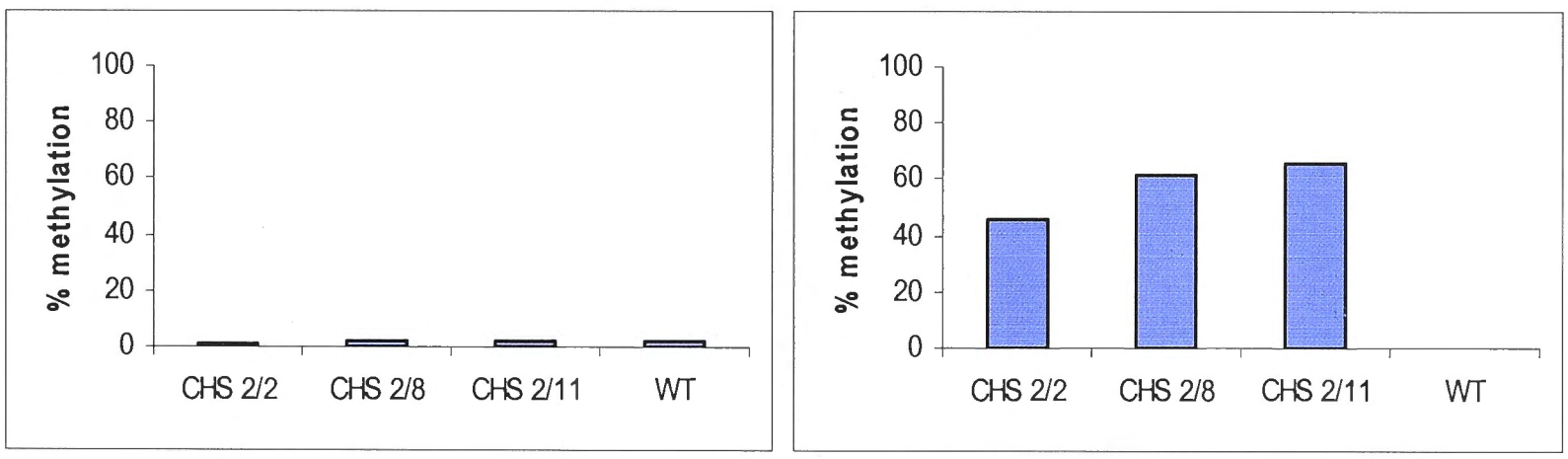
B: CHS2 Plant lines



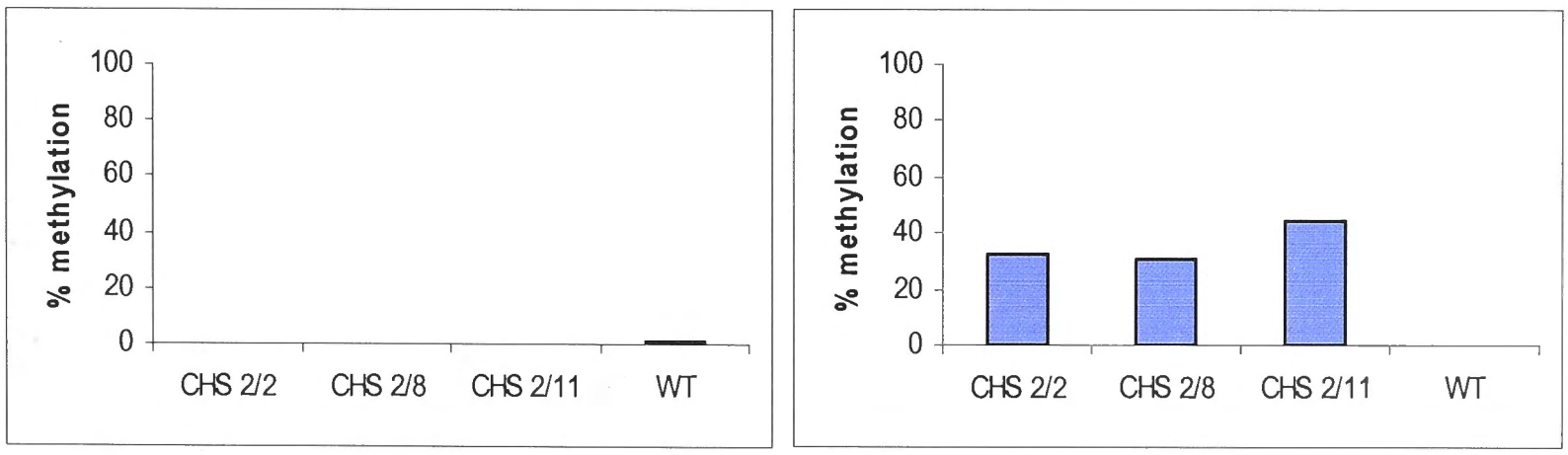
I CG methylation



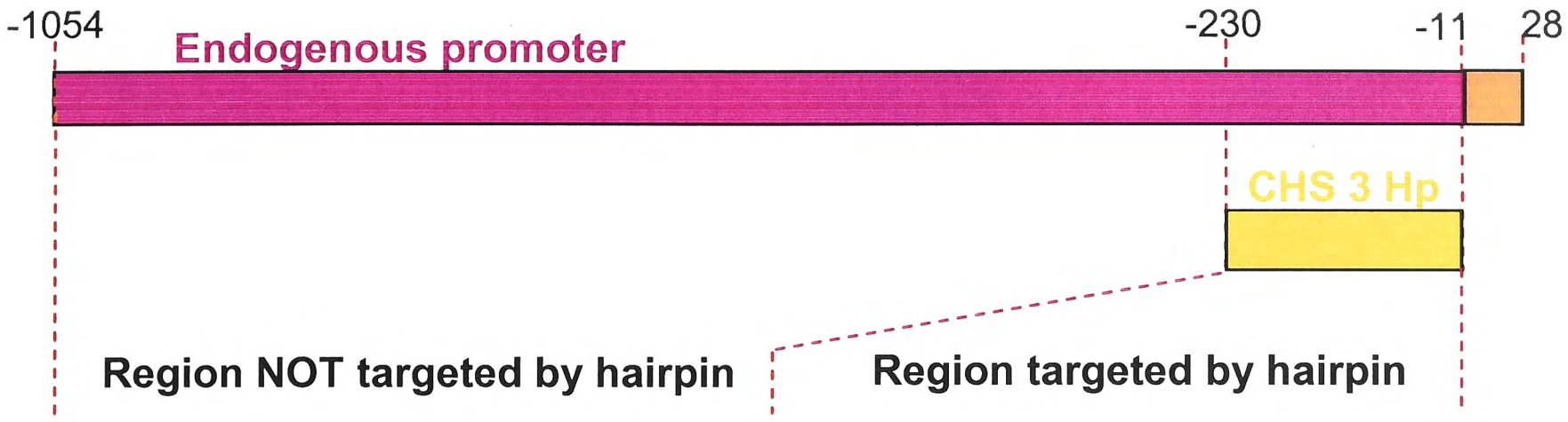
II CnG methylation



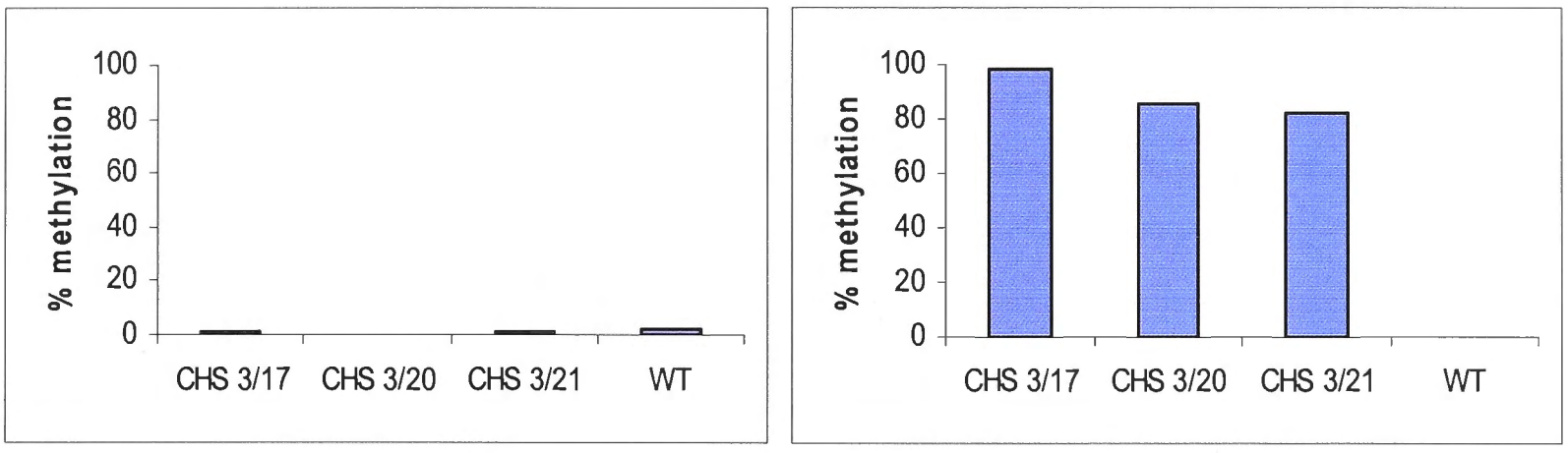
III non-symmetric methylation



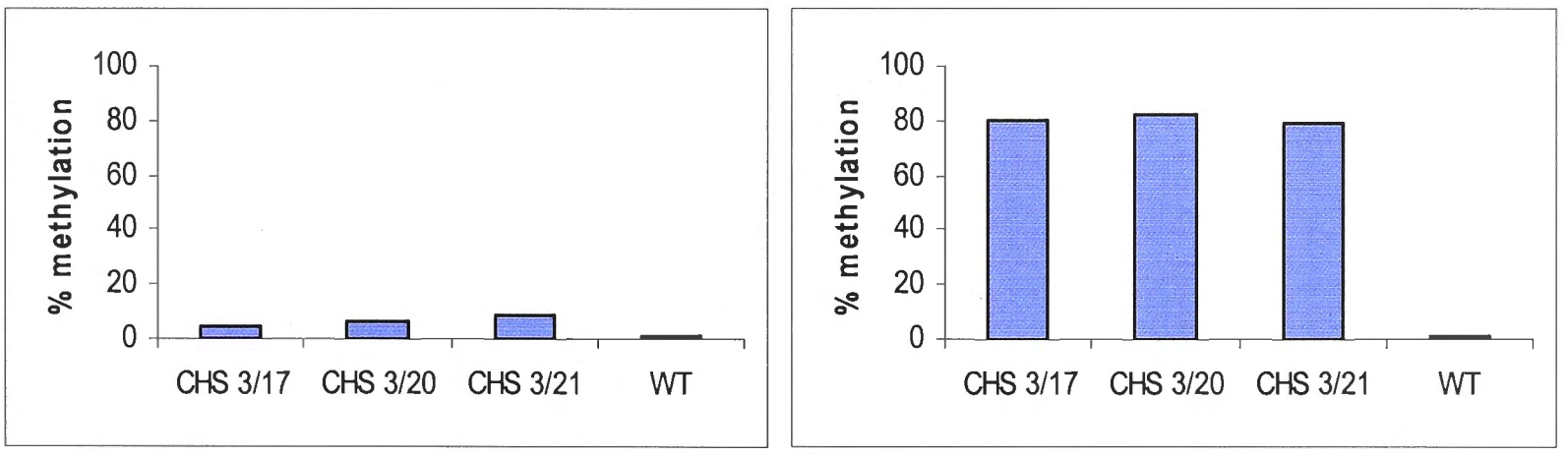
C: CHS3 Plant lines



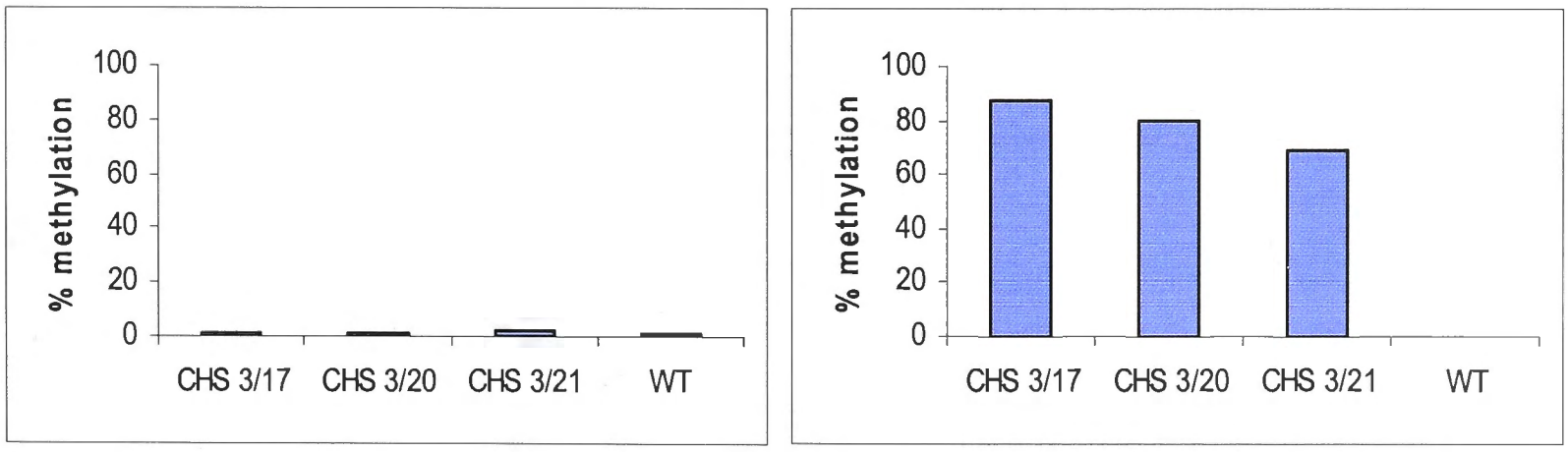
I CG methylation



II CnG methylation



III non-symmetric methylation



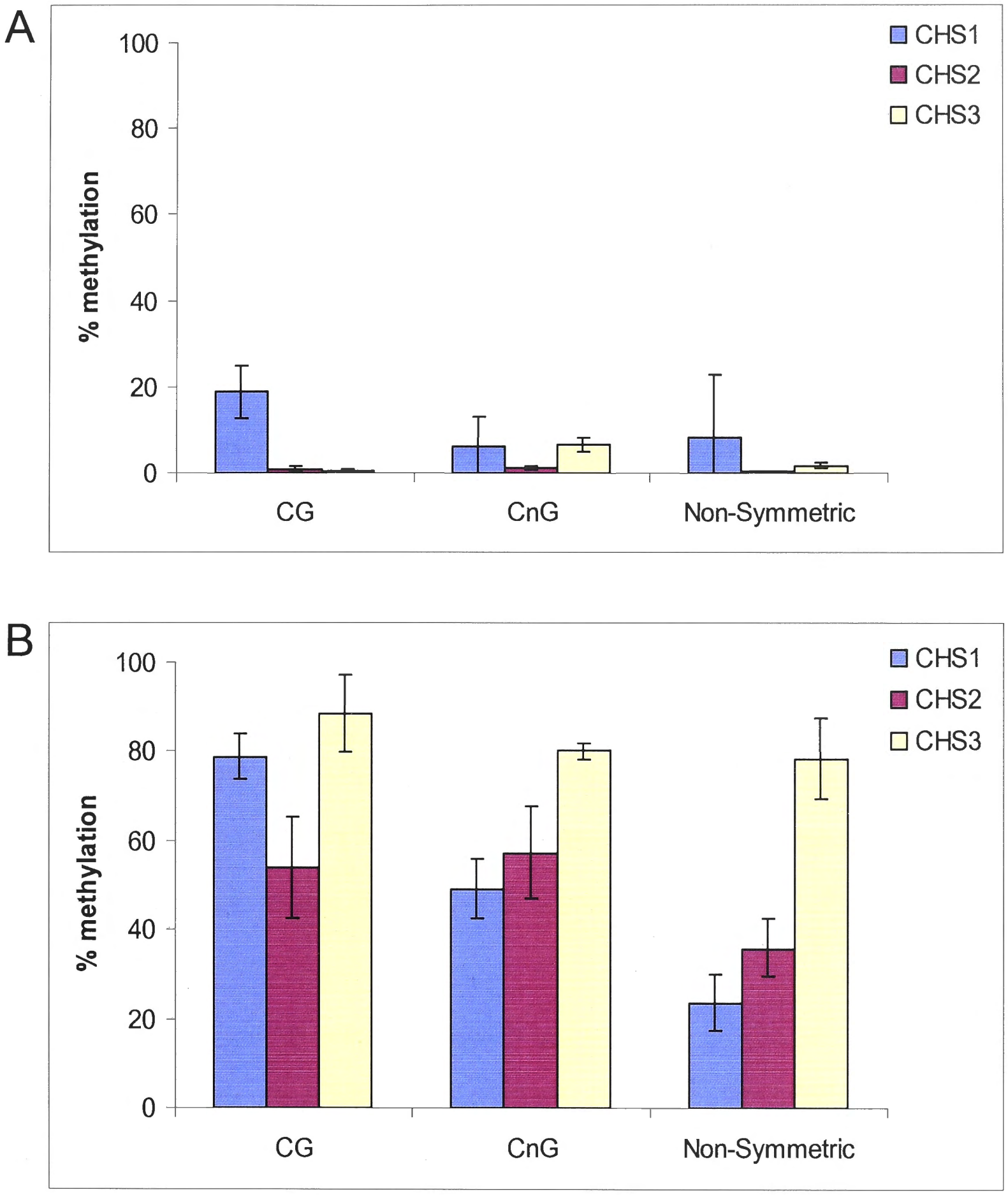


Figure 4.22: Methylation levels of the endogenous *CHS* gene in plant lines carrying CHS hpRNA constructs targeting the promoter region of the *CHS* gene.

A Methylation levels detected in sections of the endogenous *CHS* gene that were not targeted by the CHS hpRNA constructs.

B Methylation levels detected in sections of the endogenous *CHS* gene that were directly targeted by the CHS hpRNA constructs.

Bars represent mean value for cytosine methylation detected in the three plant lines that carried CHS1, CHS2 or CHS3 hpRNA construct. Error bars represent standard deviation around the mean.

observed in plant lines carrying CHS2 or CHS3 constructs. This is probably due to the fact that only a short segment of sequence that is not targeted by the CHS Hp transgene was analysed in plant lines carrying the CHS1 construct (Fig. 4.21). The analysed sequence was also in close proximity to the sequence targeted by the transgene, and the methylation in this segment could have been due to the spread of methylation from the targeted area (see section 4.3.2.3).

In all the CHS1, CHS2 and CHS3 plant lines analysed, a higher percentage of methylation was observed for cytosine residues located in symmetric sequences (CG or CnG) than those in a non-symmetric sequences (Fig. 4.21, Fig. 4.22). Furthermore, the levels of CG methylation were higher than levels of CnG methylation in most plant lines. It is interesting to note that plants carrying the shortest silencing construct (CHS3) had the highest level of symmetric and non-symmetric methylation. The methylation levels were consistently high for all three plant lines carrying the CHS3 silencing construct. Lower methylation levels and more variation between plant lines were observed in plants carrying the CHS2 or CHS3 constructs (Fig. 4.21, Fig. 4.22).

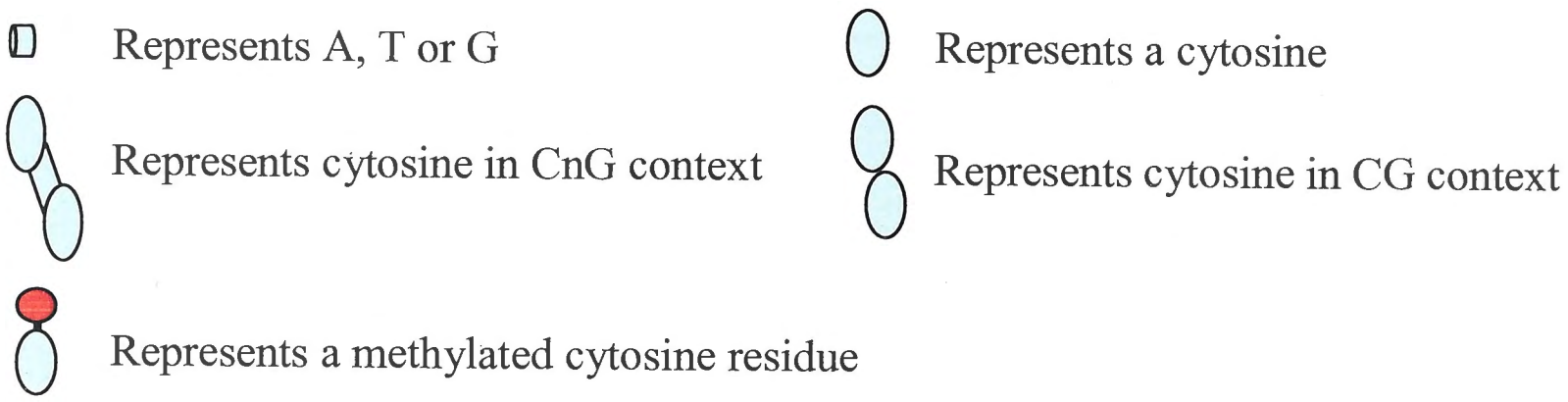
4.3.2.3 Spread of methylation out of the targeted region

To determine whether methylation was confined strictly to the targeted region, methylation of individual cytosine residues within the targeted regions as well as regions adjacent to the area targeted by the CHS Hp transgenes was analysed. To simplify the presentation of results, I will focus on data obtained from plant lines carrying the shortest silencing construct (CHS3). Data obtained from plant lines carrying CHS2 and CHS1 constructs show similar trends and can be found in Appendix 2.

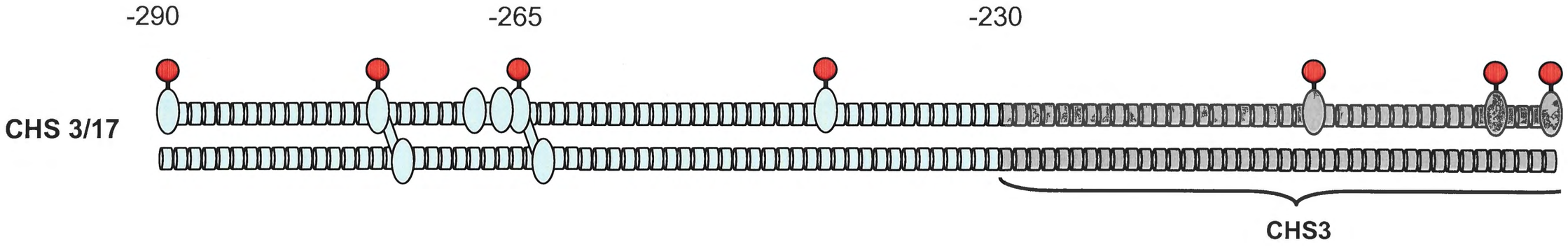
Analysis of the top strand of the endogenous CHS gene region targeted by the CHS3 hpRNA construct revealed that over 80% of cytosines in these regions were methylated (Fig. 4.21C). In addition, both symmetric and non-symmetric methylation appears to have spread in the 5' direction from the targeted region (Fig. 4.23, Fig. 4.24 and Fig. 4.25). For symmetric methylation the limit of this 5' spread appears to be at least 50bp from the target/non-target border, while for non-symmetric methylation it appears to be at least 100bp from the target/non-target border (Fig. 4.24). However, in some plant lines a low percentage of cytosine methylation appears at even greater distance from the target/non-target border. For example, in the plant line CHS 3/17, 5% of symmetric cytosines are methylated even in the region that is 190bp from the targeted area.

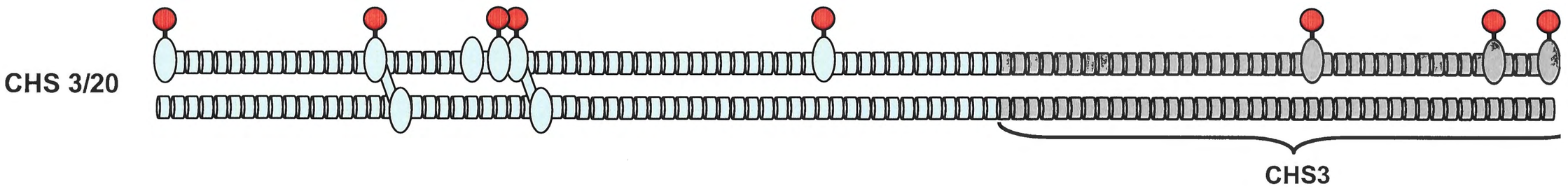
The levels of non-symmetric methylation in non-targeted regions decrease more slowly than the levels of symmetric methylation. For both symmetric and non-symmetric methylation, the percentage of methylated cytosines gradually drops and eventually approaches the baseline level observed in wild type plants. In two out of three analysed plant lines, symmetric methylation reaches the baseline level at approximately 50bp from the last cytosine residue that was targeted by the CHS3 hpRNA silencing construct (Fig. 4.24). Similarly, in two out of three analysed plant lines, levels of non-symmetric methylation fall below 10% at approximately 100bp from the last targeted cytosine residue (Fig. 4.25). For all analysed plant lines the non-symmetric methylation eventually reaches the baseline level at approximately 190bp from the last targeted cytosine residue.

Figure 4.23: Schematic representation of spread of methylation observed in plant lines carrying CHS3 hpRNA constructs targeting promoter region of the endogenous *CHS* gene.



Sections directly targeted by a hpRNA transgene are coloured grey. Sections of promoter that were not directly targeted are coloured blue.


-290
-265
-230
CHS 3/17
CHS3


CHS 3/20
CHS3

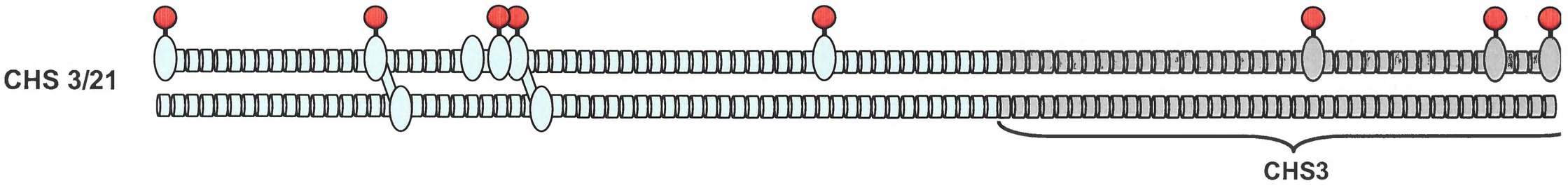

CHS 3/21
CHS3

Fig. 4.24: Analysis of methylation of individual symmetric (CG or CnG) cytosine residues in plant lines carrying CHS3 hpRNA transgene.

Seed from plant lines 3/17, 3/20 and 3/21 was germinated on MS medium containing Kanamycin. WT seed was germinated at the same time, but on MS medium that did not contain Kanamycin selection.

Two weeks after the germination leaf tissue was collected from at least five plants from each of the analysed plant lines. DNA was extracted, treated with sodium bisulfite and methylation analysed as described in section 4.2.11.

The pink bar represents the endogenous *CHS* promoter. The yellow bar represents CHS3 hpRNA transgenes.

At least 20 sequences were obtained for each plant line. The percentage of methylation reflects the number of sequences (out of a total of 20) in which a particular cytosine residue was methylated.

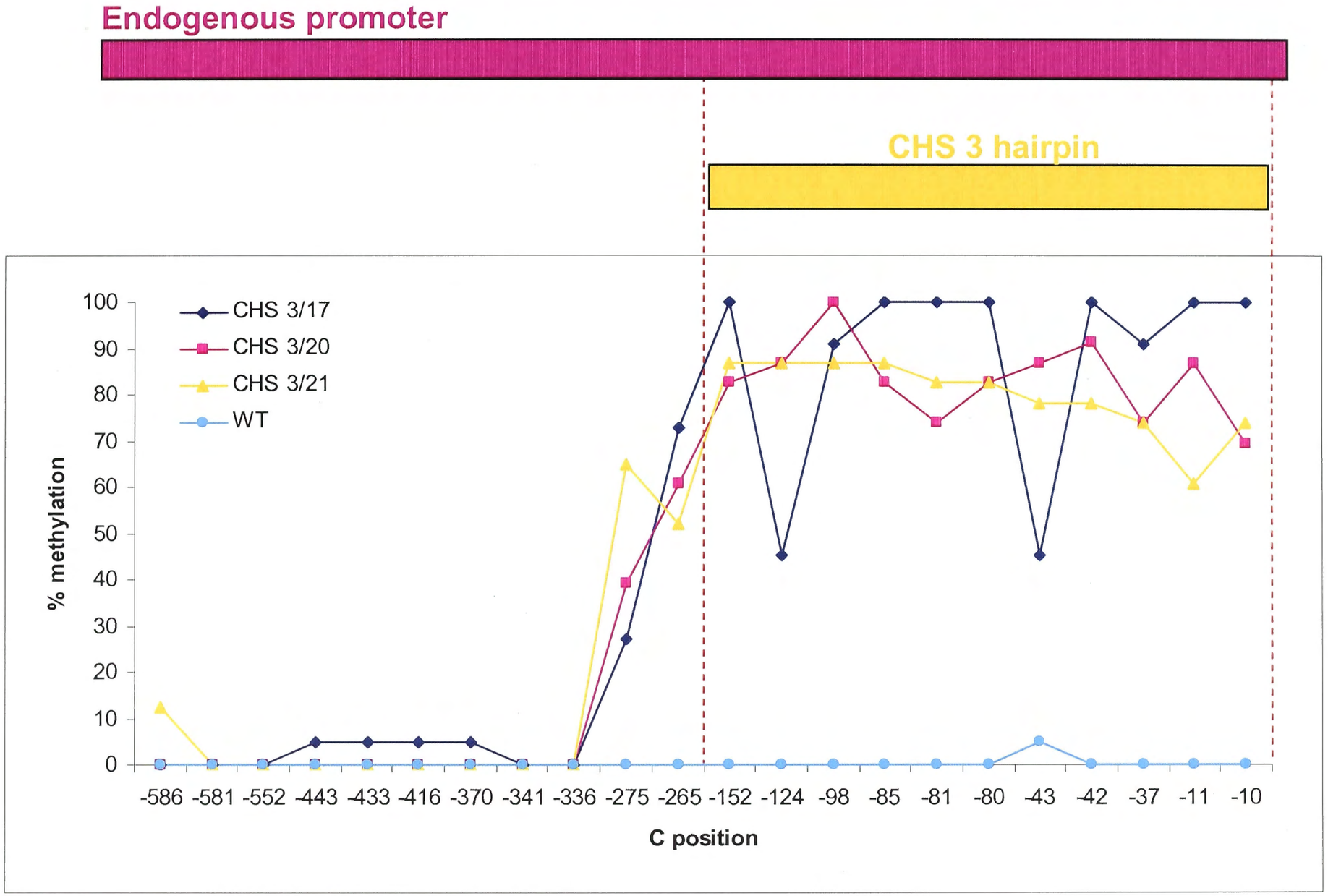

Endogenous promoter
CHS 3 hairpin
CHS 3/17
CHS 3/20
CHS 3/21
WT
% methylation
100
90
80
70
60
50
40
30
20
10
0
-586 -581 -552 -443 -433 -416 -370 -341 -336 -275 -265 -152 -124 -98 -85 -81 -80 -43 -42 -37 -11 -10
C position

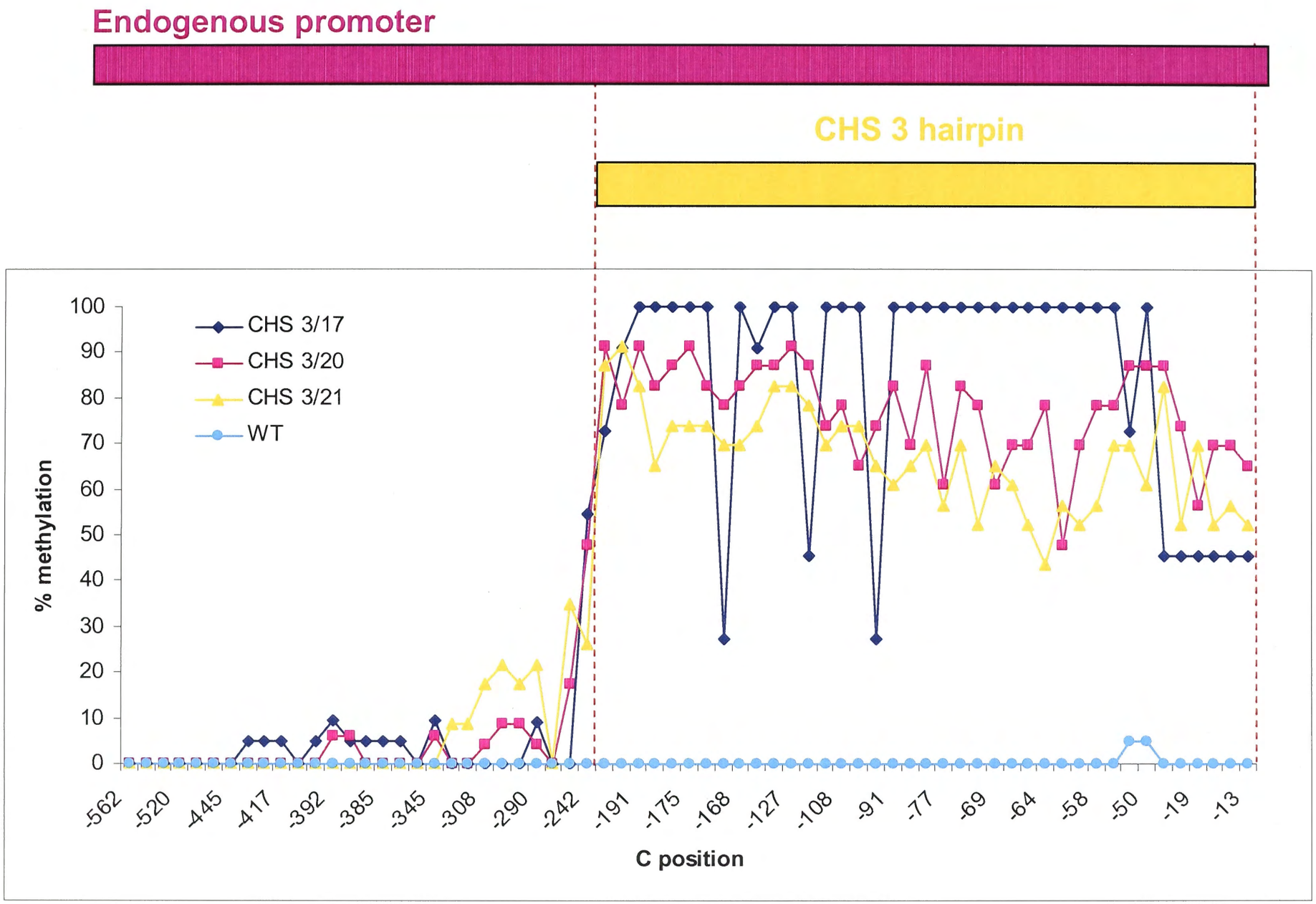
Fig. 4.25: Analysis of methylation of individual non-symmetric cytosine residues in plant lines carrying CHS3 hpRNA transgene.

Seed from plant lines 3/17, 3/20 and 3/21 was germinated on MS medium containing Kanamycin. WT seed was germinated at the same time, but on MS medium that did not contain Kanamycin selection.

Two weeks after the germination leaf tissue was collected from at least five plants from each of the analysed plant lines. DNA was extracted, treated with sodium bisulfite and methylation analysed as described in section 4.2.11.

The pink bar represents the endogenous *CHS* promoter. The yellow bar represents CHS3 hpRNA transgenes.

At least 20 sequences were obtained for each plant line. The percentage of methylation reflects the number of sequences (out of a total of 20) in which a particular cytosine residue was methylated.


Endogenous promoter
CHS 3 hairpin
CHS 3/17
CHS 3/20
CHS 3/21
WT
% methylation
C position
100
90
80
70
60
50
40
30
20
10
0
-562
-520
-445
-417
-392
-385
-345
-308
-290
-242
-191
-175
-168
-127
-108
-91
-77
-69
-64
-58
-50
-19
-13

An analysis of methylation of individual cytosine residues revealed that although the 3 lines (CHS3/17, CHS3/20 and CHS3/21) had very similar levels of overall methylation the pattern of methylation of individual cytosines in these lines is different (Fig. 4.24 and Fig.4.25). The differences in the silencing phenotypes could be explained by these differences. For example, lines with similar overall methylation levels could have different regions of promoter methylated resulting in different effects on transcription and efficiency of TGS.

4.3.3 DNA methylation patterns associated with PTGS of CHS

The plant lines used to investigate the methylation patterns in plants displaying post transcriptional silencing of the *CHS* gene have been previously described by Wesley *et al.* (2001). I used two different plant lines displaying PTGS of chalcone synthase. One line carried a 100bp CHS Hp construct (CHS100), while the second carried a 400bp construct (CHS400). Bisulfite sequencing was again used to analyse methylation patterns in these plant lines. The positions of CHS Hp silencing constructs and expected Bisulfite PCR products are outlined in Fig. 4.26.

Again, depending on the plant line being analysed and the primer pair being used, bisulfite PCR products could be amplified either from both the endogenous gene and the transgene; or from the endogenous gene only (Fig. 4.27 and Fig. 4.28). However, in this case, the presence of an intron allowed me to clearly distinguish between products amplified from the endogenous gene and those amplified from the transgene. Consequently, methylation levels of the endogenous gene and those of the transgene could be compared directly.

Figure 4.26: Schematic representation of the *CHS* coding region, hpRNA transgenes and the expected amplification products from the bisulfite PCR.

The pink bar represents the coding region of the endogenous *CHS* gene. The yellow section of this bar represents the intron in the endogenous *CHS* gene. The orange section represents the promoter of the endogenous *CHS* gene.

The blue lines with arrows mark regions of the *CHS* gene amplified by specific sets of primers. Each amplification product was named according to the set of primers used for the amplification.

The green and black bars represent CHS100 and CHS400 hpRNA transgenes, respectively.

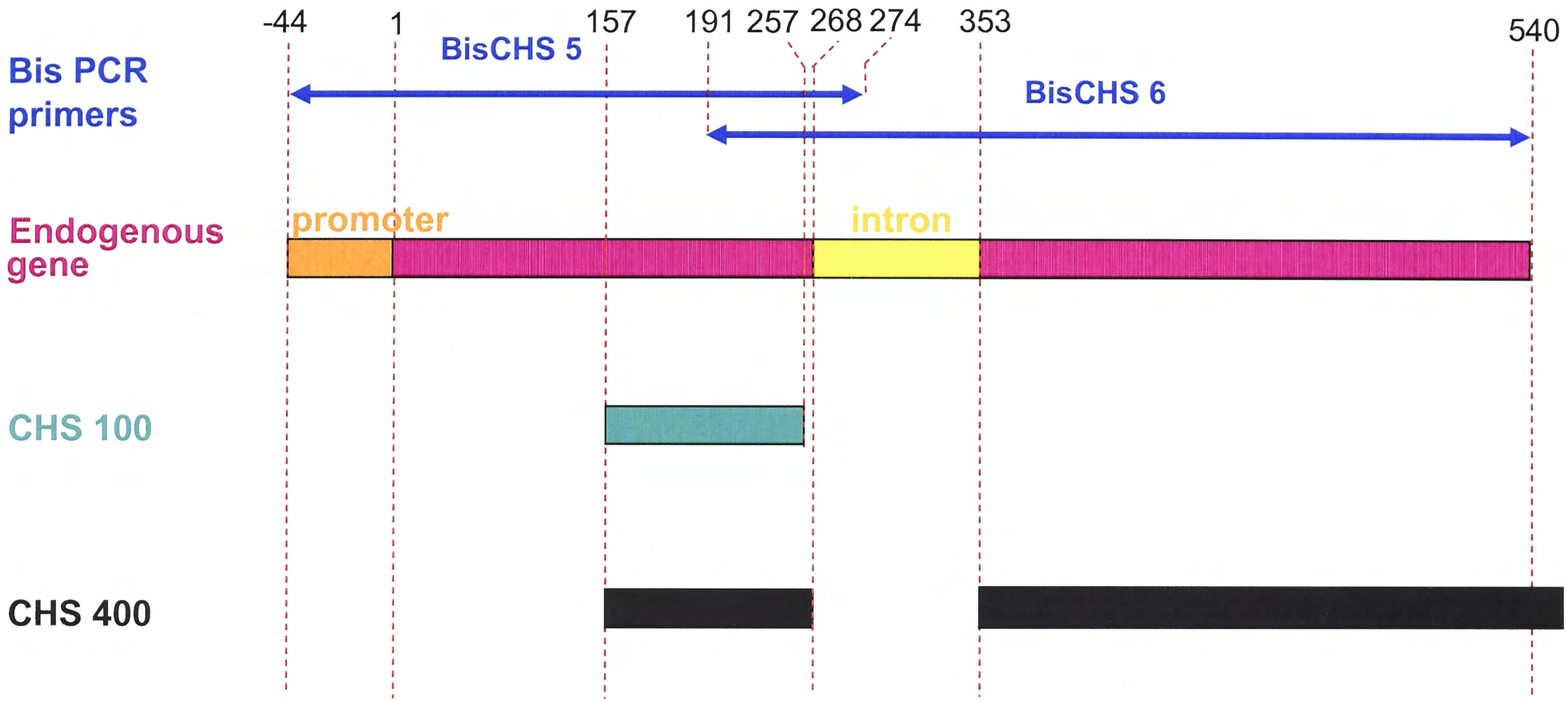

-44
1
157
191
257
268
274
353
540
BisCHS 5
BisCHS 6
Bis PCR primers
Endogenous gene
promoter
intron
CHS 100
CHS 400

Figure 4.27: Schematic representation of bisulfite PCR products obtained by amplification from DNA extracted from plant lines carrying CHS 100 constructs.

The pink bar represents the coding region of the endogenous *CHS* gene. The yellow section of this bar represents the intron in the endogenous *CHS* gene. The orange section represents the promoter of the endogenous *CHS* gene.

The blue lines with arrows mark regions of the *CHS* gene amplified by specific sets of primers. Each amplification product was named according to the set of primers used for the amplification.

The green bar represents the CHS 100 hpRNA transgene.

The pink lines represent bisulfite PCR products. The orange section within these lines represents the sequence amplified from the promoter of the endogenous *CHS* gene. The yellow section within these lines represents the sequence amplified from the intronic region of the endogenous *CHS* gene.

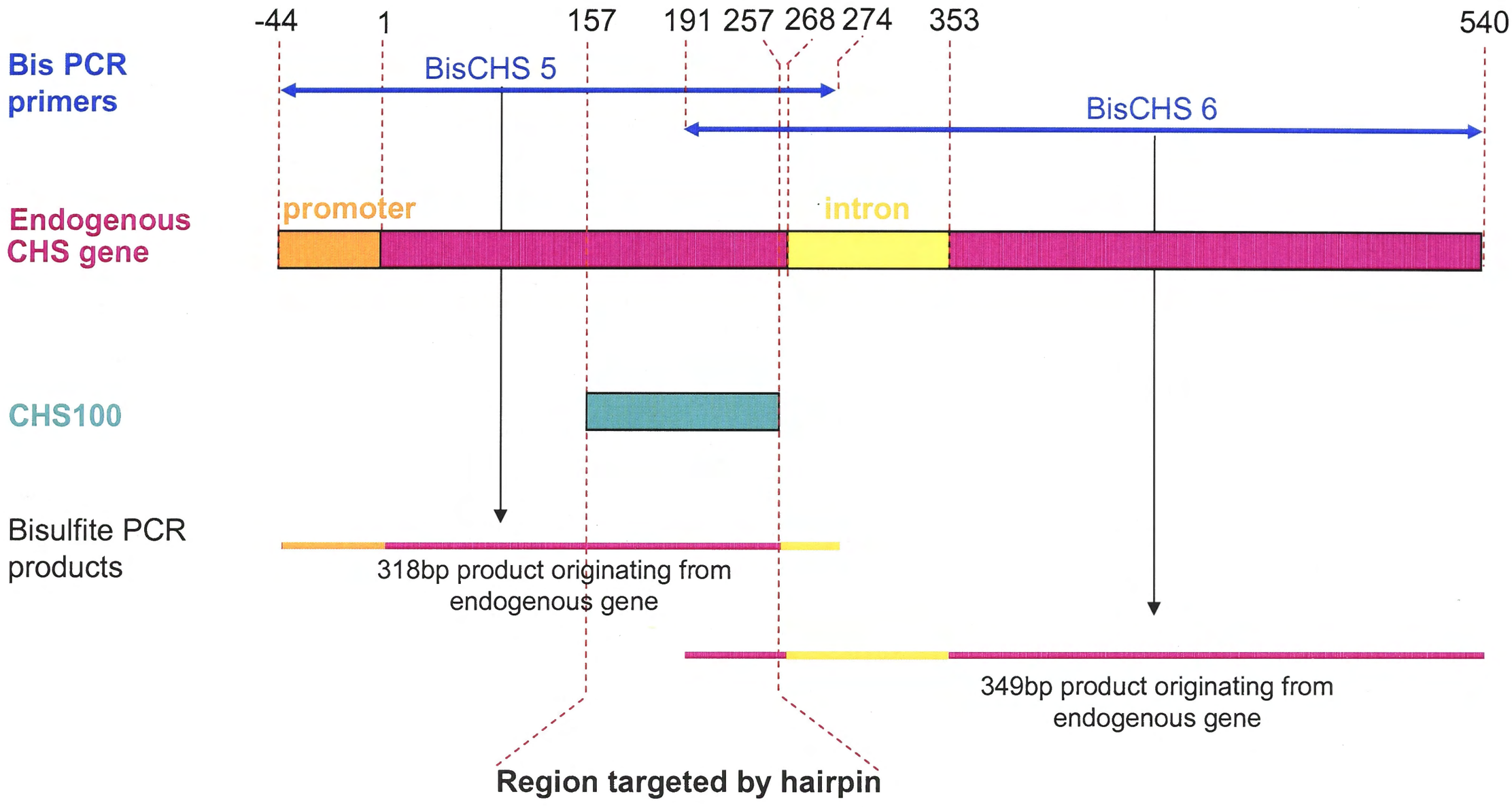

-44
1
157
191
257
268
274
353
540
Bis PCR primers
BisCHS 5
BisCHS 6
Endogenous CHS gene
promoter
intron
CHS100
Bisulfite PCR products
318bp product originating from endogenous gene
349bp product originating from endogenous gene
Region targeted by hairpin

Figure 4.28: Schematic representation of bisulfite PCR products obtained by amplification from DNA extracted from plant lines carrying CHS 400 constructs.

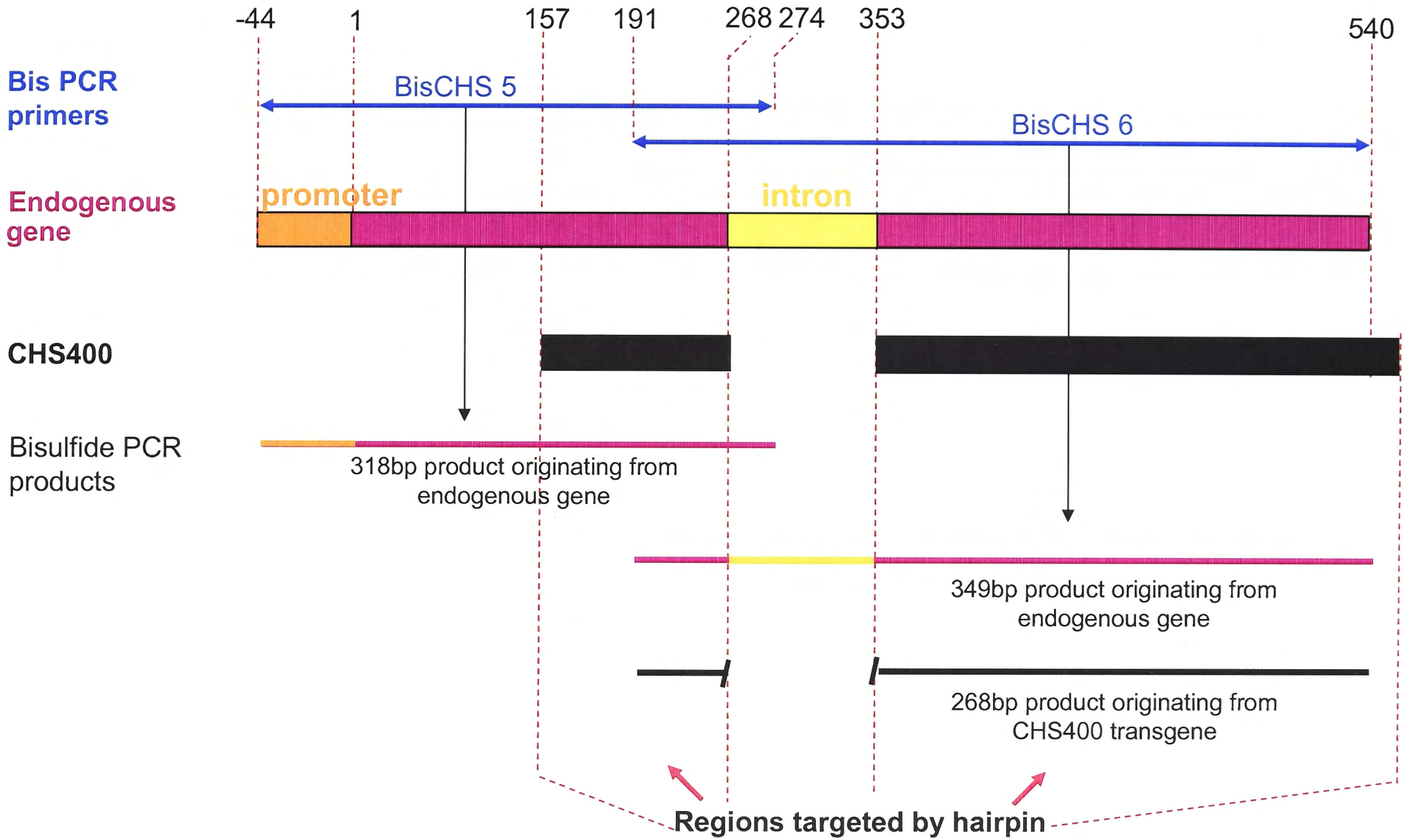
The pink bar represents the coding region of the endogenous *CHS* gene. The yellow section of this bar represents the intron in the endogenous *CHS* gene. The orange section represents the promoter of the endogenous *CHS* gene.

The blue lines with arrows mark regions of the *CHS* gene amplified by specific sets of primers. Each amplification product was named according to the set of primers used for the amplification.

The black bar represents CHS 400 hpRNA transgene.

The pink lines represent bisulfite PCR products. The orange section within these lines represents the sequence amplified from the promoter of the endogenous *CHS* gene. The yellow section within these lines represents the sequence amplified from the intronic region of the endogenous *CHS* gene.

The black line represents the bisulfite PCR product that was amplified using the CHS 400 hpRNA transgene as a template. As the transgene did not contain the intronic region present in the endogenous *CHS* gene this product was shorter (268bp) than the product generated using the endogenous *CHS* gene as a template (349bp).


-44
1
157
191
268
274
353
540
Bis PCR primers
BisCHS 5
BisCHS 6
Endogenous gene
promoter
intron
CHS400
Bisulfide PCR products
318bp product originating from endogenous gene
349bp product originating from endogenous gene
268bp product originating from CHS400 transgene
Regions targeted by hairpin

4.3.3.1 Levels of transgene and endogenous gene methylation

When DNA extracted from plant lines carrying the CHS100 silencing construct was analysed by bisulfite sequencing both BisCHS5 and BisCHS6 products could only be amplified from the endogenous *CHS* gene (Fig. 4.27). Similarly, when DNA extracted from plant lines carrying the CHS400 silencing construct was used, the BisCHS5 PCR product could only be amplified from the endogenous gene. However, a BisCHS6 product could be amplified from either the endogenous gene or the transgene. The CHS400 silencing construct was designed to target *CHS* RNA and therefore did not contain the intron which is present in the genomic sequence of the *CHS* gene. Consequently, when the BisCHS6 product was generated by amplification from the CHS400 transgene it yielded a 268bp product, but when the endogenous gene was used as a template a 349bp product was generated (Fig. 4.28). These products were separated by electrophoresis and individually analysed.

An analysis of overall methylation levels of the endogenous gene and the CHS400 transgene in plant lines carrying the CHS400 silencing construct revealed that the transgene had higher methylation levels than the endogenous gene (Fig. 4.29). In particular, 80% of cytosines in CG dinucleotides were methylated in the transgene while in the endogenous gene only 40% of CG cytosines were methylated. Furthermore, in the endogenous gene, the 5' portion of the analysed region was found to have a higher percentage of methylated cytosines than the 3' end. In contrast, in the transgene the 3'portion of the analysed region had somewhat higher methylation levels than the 5' section (Fig. 4.29).

Fig. 4.29: Comparison of methylation levels present in the hpRNA transgene and the endogenous *CHS* gene in plants carrying the CHS400 hpRNA transgene.

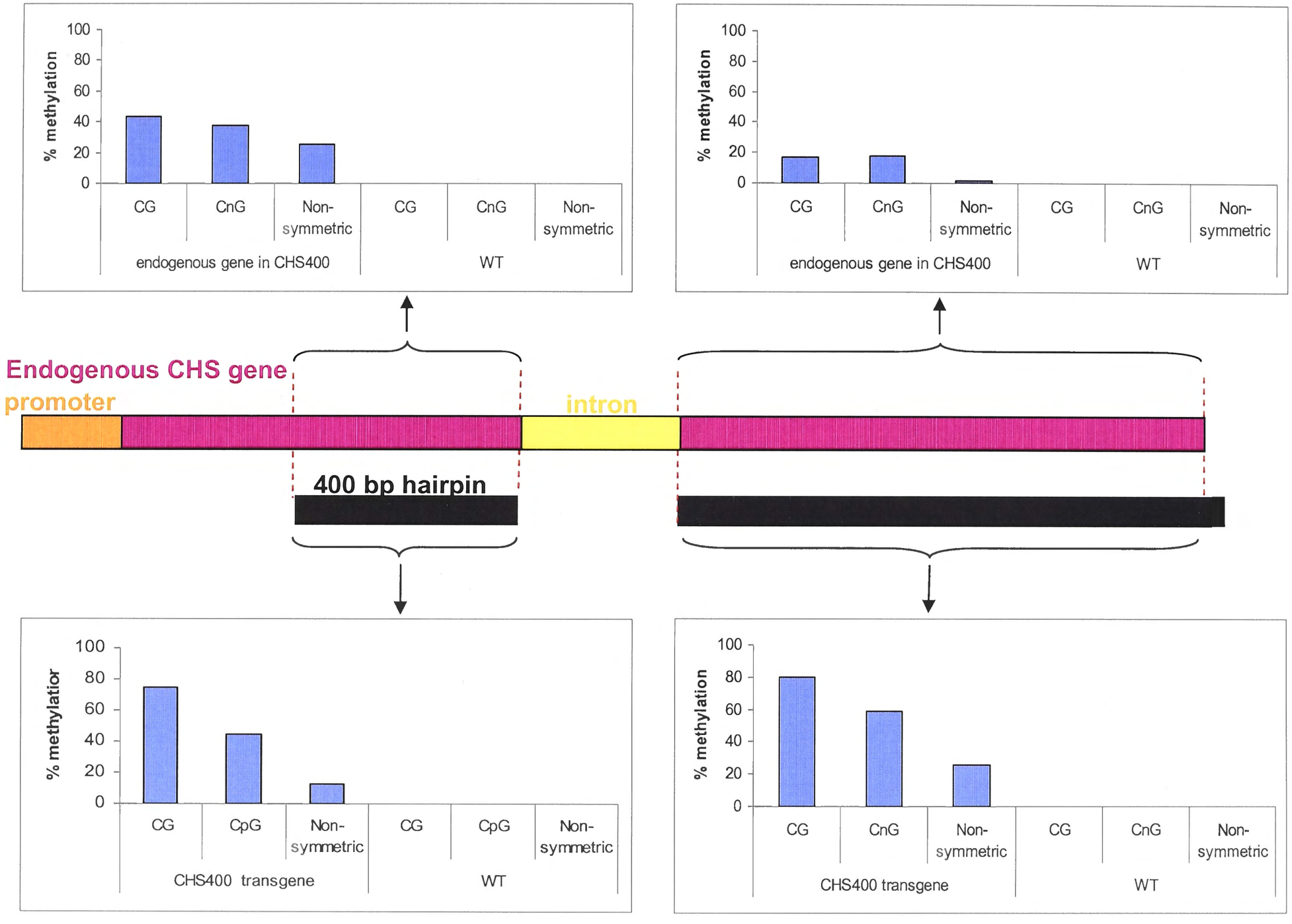
Seed from a plant line carrying the CHS400 hpRNA transgene was germinated on MS medium containing Kanamycin. WT seed was germinated at the same time, but on MS medium that did not contain Kanamycin selection.

Two weeks after germination, leaf tissue was collected from at least five individual plants of each type (CHS400 carrying plants and WT plants). DNA was extracted, treated with sodium bisulfite and the methylation status analysed as described in section 4.2.11.

In the DNA extracted from plants carrying the CHS400 hpRNA transgene there were two possible templates for amplification: the CHS400 hpRNA transgene and the endogenous *CHS* gene. Because the CHS400 hpRNA transgene did not contain the intronic region present in the endogenous *CHS* gene, the bisulfite PCR product amplified from the transgene template was shorter (268bp) than the product generated using the endogenous *CHS* gene as a template (349bp). This difference in size allowed the two products to be separated using gel electrophoresis and individually analysed for methylation levels.

The percentage of methylation for each of the plant lines was calculated as outlined in table 4.5.

Methylation levels were calculated for three different types of cytosine residues: those adjacent to a guanine residue (CG); those 5' of guanine residue but separated by another residue (CnG) and those that did not fall in one of the previous two categories (non-symmetric cytosine residues).


% methylation
100
80
60
40
20
0
CG
CnG
Non-symmetric
CG
CnG
Non-symmetric
endogenous gene in CHS400
WT
Endogenous CHS gene
promoter
intron
400 bp hairpin
% methylation
100
80
60
40
20
0
CG
CnG
Non-symmetric
CG
CnG
Non-symmetric
endogenous gene in CHS400
WT
% methylation
100
80
60
40
20
0
CG
CpG
Non-symmetric
CG
CpG
Non-symmetric
CHS400 transgene
WT
% methylation
100
80
60
40
20
0
CG
CnG
Non-symmetric
CG
CnG
Non-symmetric
CHS400 transgene
WT

It is interesting to note that in plant lines carrying CHS Hp constructs which targeted promoter regions, the endogenous gene appears to acquire higher methylation levels than the CHS Hp transgenes. In plants carrying CHS Hp constructs that targeted transcribed regions this situation was reversed as the transgene acquired considerably higher methylation levels than the endogenous gene.

4.3.3.2 Comparison of methylation levels in regions targeted by the CHS Hp transgenes and regions that were not targeted by the CHS Hp transgenes

An analysis of methylation for regions of the endogenous *CHS* gene targeted by the CHS100 Hp and CHS400 Hp transgenes revealed that, in these regions, between 20% and 40% of cytosines were methylated (Fig. 4.30). Slightly higher methylation levels were observed in the plant line carrying the CHS400 Hp construct, particularly with CG cytosines in the 5' portion of the endogenous gene target. Very low levels of methylation were observed in the regions of the endogenous gene that were not targeted by the *CHS* silencing constructs. In the plant line carrying the CHS400 Hp construct, the intronic region, which was not directly targeted by the CHS400 Hp, had a higher level of methylation than the other non-targeted regions. This suggests that methylation has spread from one, or both, of the adjacent targeted regions (see section 4.3.3.3).

Overall, the methylation levels observed in the regions targeted by the CHS100 and CHS400 constructs were much lower than the methylation levels observed in the promoter regions targeted by similar constructs (Fig. 4.30 and Fig. 4.21). Perhaps this difference could be due to the presence of a mechanism which protects transcribed regions from methylation while allowing the non-transcribed regions to be methylated.

Fig. 4.30: Comparison of methylation levels present in sections of the endogenous *CHS* gene homologous to the hpRNA transgenes and sections that do not share homology with the hpRNA transgenes.

Seed from plant lines carrying either CHS100 or CHS400 hpRNA transgene was germinated on MS medium containing Kanamycin. WT seed was germinated at the same time, but on MS medium that did not contain Kanamycin selection.

Two weeks after germination, leaf tissue was collected from at least five individual plants from each plant line. DNA was extracted, treated with sodium bisulfite and methylation analysed as described in section 4.2.11.

The pink bar represents the coding region of the endogenous *CHS* gene. The yellow section of this bar represents the intronic of the endogenous *CHS* gene. The orange section represents the promoter of the endogenous *CHS* gene.

The green bar and the black bar represent CHS100 and CHS400 hpRNA transgenes, respectively. Bars representing transgenes are positioned parallel to the sections of the endogenous gene from which the transgene sequence was derived.

The bar representing the endogenous *CHS* gene is divided into sections that are homologous to the transgene (marked with red brackets) and sections that do not have homology to the transgene (marked with black brackets).

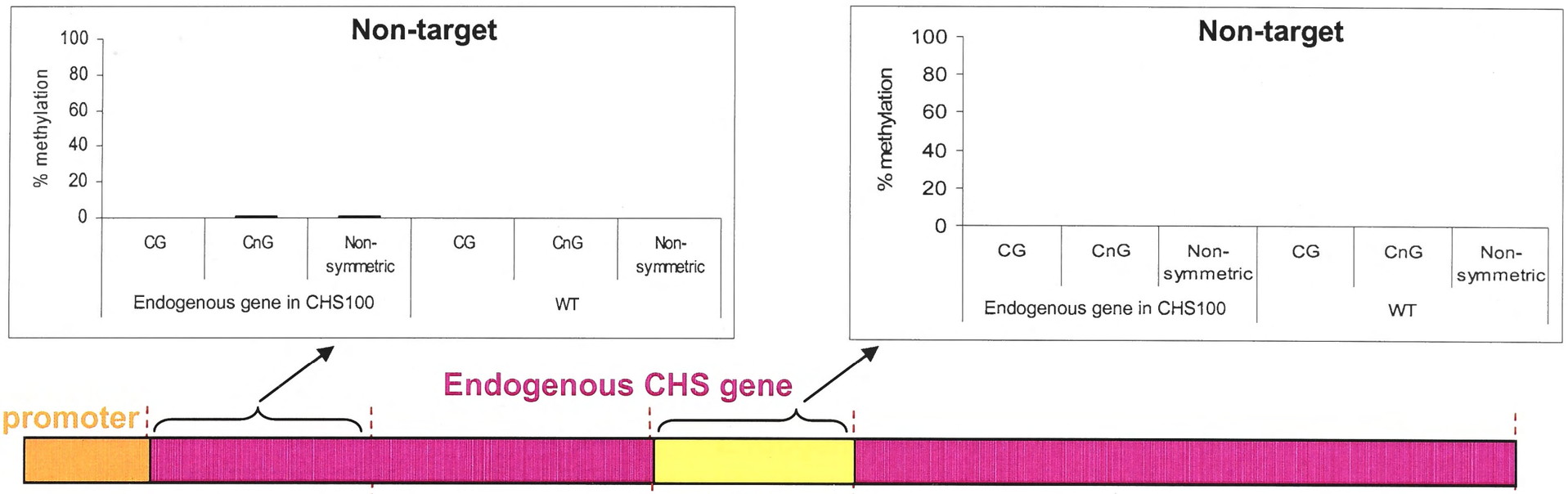
The percentage of methylation for each of the bracketed sections of the endogenous *CHS* gene was calculated as outlined in table 4.5.

The methylation levels were calculated for three different types of cytosine residues: those adjacent to a guanine residue (CG); those 5' of guanine residue but separated by another residue (CnG) and those that did not fall in one of the previous two categories (non-symmetric cytosine residues).

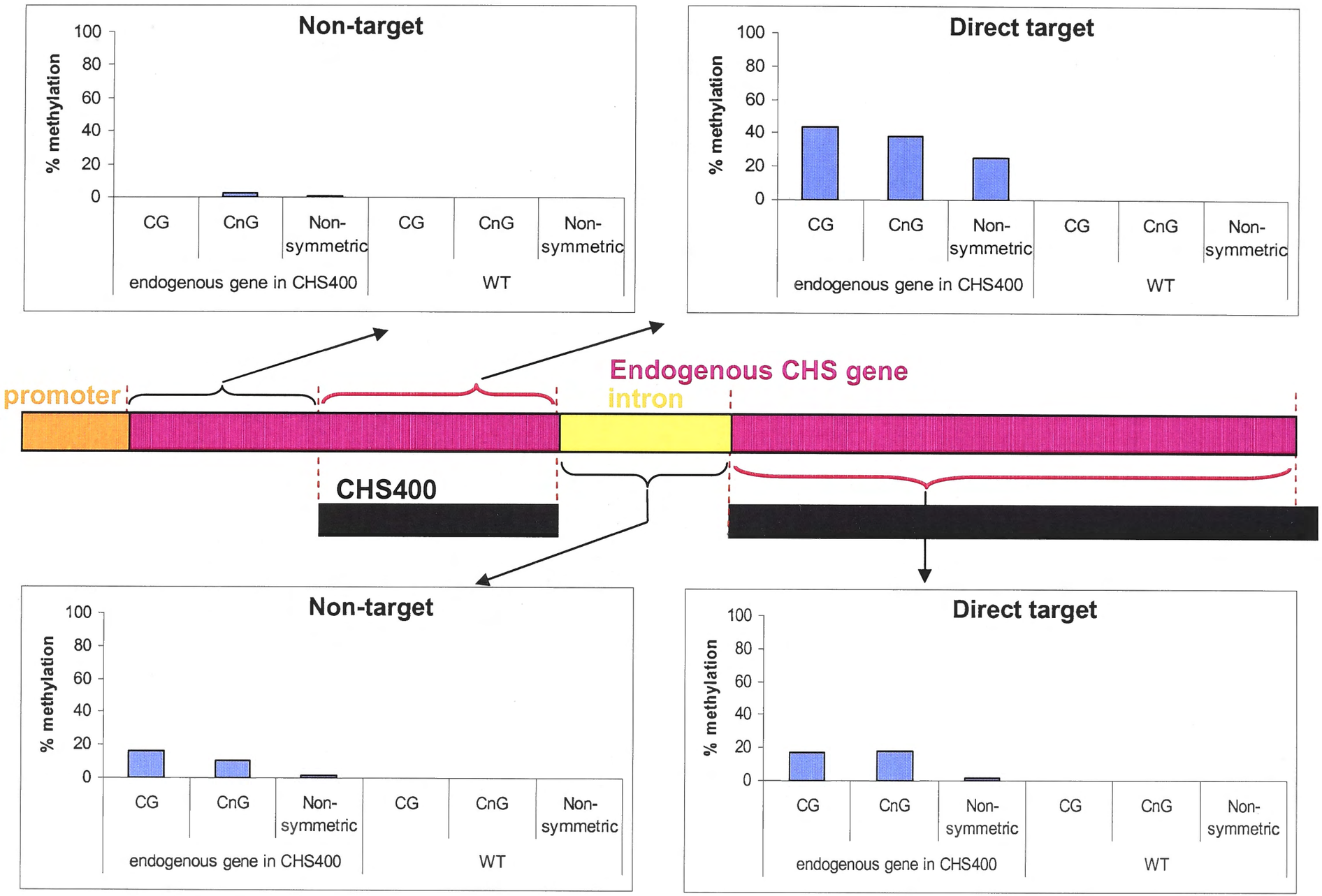
A: Comparison of methylation levels present in the regions of the endogenous *CHS* gene homologous to the CHS100 hpRNA transgene and regions that do not share homology with the transgene.

B: Comparison of methylation levels present in the regions of the endogenous *CHS* gene homologous to the CHS400 hpRNA transgene and regions that do not share homology with the transgene.

A


Non-target
% methylation
100
80
60
40
20
0
CG
CnG
Non-symmetric
CG
CnG
Non-symmetric
Endogenous gene in CHS100
WT
Non-target
% methylation
100
80
60
40
20
0
CG
CnG
Non-symmetric
CG
CnG
Non-symmetric
Endogenous gene in CHS100
WT
Endogenous CHS gene
promoter
intron
CHS100
Direct target
% methylation
100
80
60
40
20
0
CG
CnG
Non-symmetric
CG
CnG
Non-symmetric
Endogenous gene in CHS100
WT
Non-target
% methylation
100
80
60
40
20
0
CG
CnG
Non-symmetric
CG
CnG
Non-symmetric
Endogenous gene in CHS100
WT

B


Non-target
% methylation
100
80
60
40
20
0
CG
CnG
Non-symmetric
CG
CnG
Non-symmetric
endogenous gene in CHS400
WT
Direct target
% methylation
100
80
60
40
20
0
CG
CnG
Non-symmetric
CG
CnG
Non-symmetric
endogenous gene in CHS400
WT
promoter
Endogenous CHS gene
intron
CHS400
Non-target
% methylation
100
80
60
40
20
0
CG
CnG
Non-symmetric
CG
CnG
Non-symmetric
endogenous gene in CHS400
WT
Direct target
% methylation
100
80
60
40
20
0
CG
CnG
Non-symmetric
CG
CnG
Non-symmetric
endogenous gene in CHS400
WT

4.3.3.3 Spread of methylation out of the targeted region

The spread of methylation into sequences adjacent to regions directly targeted by the CHS100 and CHS400 hpRNA constructs could indicate the presence of an amplification step in the PTGS pathway (see section 4.1.2). To determine whether the spread of methylation occurred, I analysed the methylation of individual cytosine residues within the targeted regions as well as regions adjacent to the area targeted by the CHS hpRNA transgenes.

Analysis of the coding strand of the endogenous *CHS* gene regions targeted by either the CHS100 or the CHS400 hpRNA transgenes revealed that 20% to 40% of the cytosines in these regions were methylated (Fig. 4.30). Slightly higher cytosine methylation levels were observed in the plant lines carrying the CHS400 hpRNA construct, particularly when in a CG context and in the 5' portion of the target region. Low, but higher than background, levels of cytosine methylation were observed in the non-target regions of the endogenous gene, particularly in the region encoding the *CHS* intron.

The pattern of cytosine methylation (Fig. 4.31, Fig. 4.32 and Fig. 4.33) in the border region surrounding the target/non-target junction is similar to that found for the target/nontarget border region for the TGS-inducing hpRNA constructs (section 4.3.2.3). There appears to be a "spreading" of methylation from the target sequence into the non-target sequence. It is especially obvious in the 5' direction (Fig. 4.31, Fig. 4.32 and Fig. 4.33). The 5' target limit for both the CHS400 hpRNA and CHS100 hpRNA is nucleotide 157 of the endogenous *CHS* gene. Despite this, methylation occurs in cytosines in the region 5' to the target, extending to cytosine at base 137,

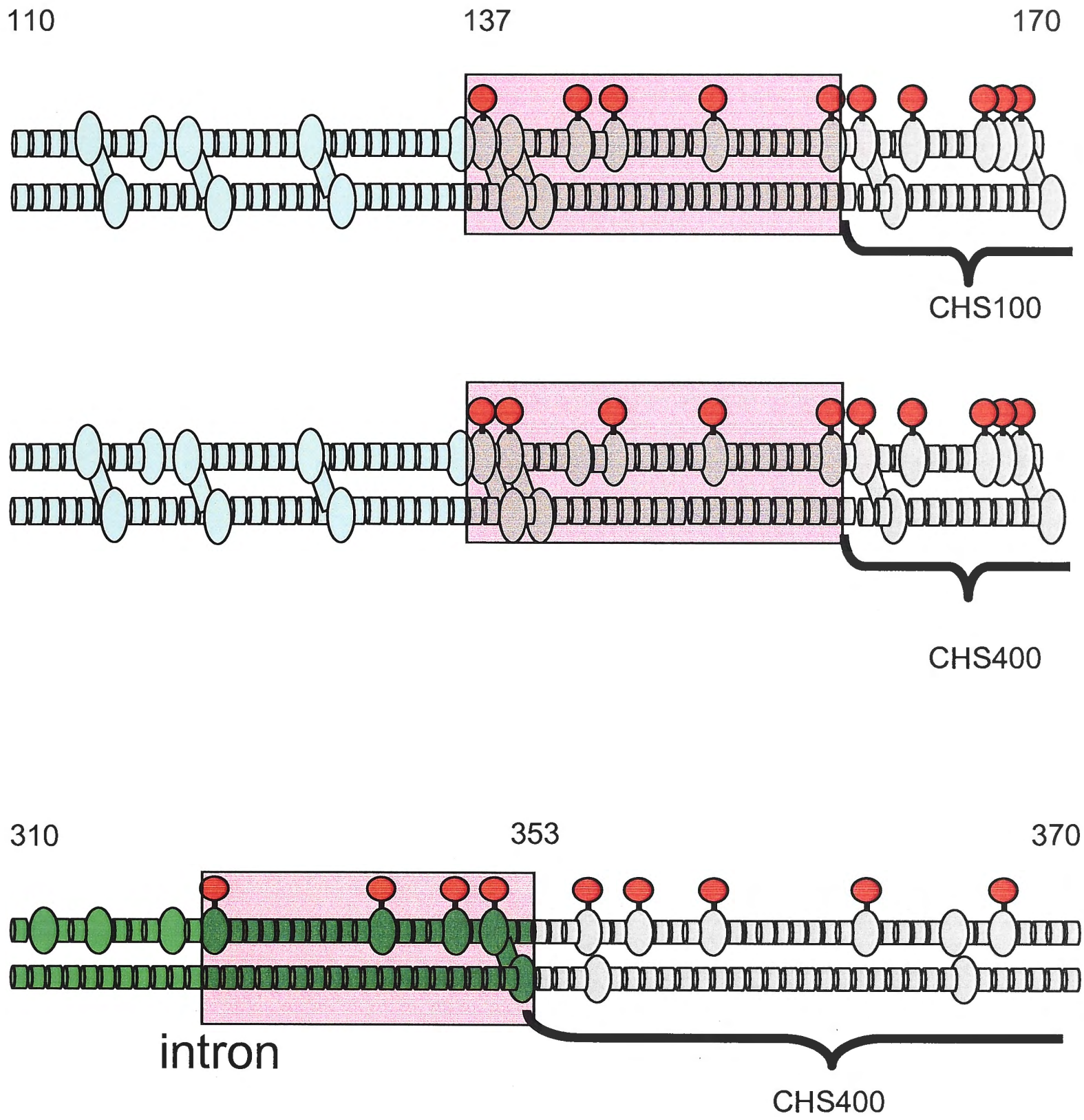


Figure 4.31: Schematic representation of spread of methylation observed in plant lines carrying hpRNA constructs targeting coding region of the endogenous *CHS* gene.

Represents A, T or G

Represents a cytosine

Represents cytosine in CnG context

Represents cytosine in CG context

Represents a methylated cytosine residue

Sections directly targeted by a hpRNA transgene are coloured grey. Exon sequences are coloured blue while the intronic region is coloured green. The area of methylation spread is framed and shadowed in pink.

Fig. 4.32: Analysis of methylation of individual symmetric (CG or CnG) cytosine residues in plant lines carrying the CHS100 or CHS400 hpRNA transgene.

Seed from plant lines CHS100 and CHS400 was germinated on MS medium containing Kanamycin. WT seed was germinated at the same time, but on MS medium that did not contain Kanamycin selection.

Two weeks after germination, leaf tissue was collected from at least five plants from each of the analysed plant lines. DNA was extracted, treated with sodium bisulfite and methylation analysed as described in section 4.2.11.

The pink bar represents the endogenous *CHS* gene. The orange section of this bar represents the promoter region while the yellow section represents the intronic region.

The green and black bars represent the CHS100 and CHS400 hpRNA transgenes, respectively. Bars representing transgenes are positioned parallel to the sections of the endogenous gene from which the transgene sequence was derived.

The blue lines with arrows represent the bisulfite PCR products used to analyse methylation patterns of the endogenous *CHS* sequences and the CHS400 hpRNA transgene. Methylation of endogenous *CHS* sequences was analysed using BisCHS5 and BisCHS6 products. Methylation of the CHS400 hpRNA transgene could only be analysed using the BisCHS6 product.

At least 20 sequences were obtained for each plant line. The percentage of methylation reflects the number of sequences (out of a total of 20) in which a particular cytosine residue was methylated.

The green line on the graph represents the methylation state of the endogenous *CHS* gene in a plant line that carries the CHS100 hpRNA transgene. The pink line on the graph represents the methylation state of the endogenous *CHS* gene in a plant line that carries the CHS400 hpRNA transgene while the black line represents the methylation state of the CHS400 hpRNA transgene in the same plant line. The gap in the black line appears because the CHS400 hpRNA transgene does not contain the intronic sequence.

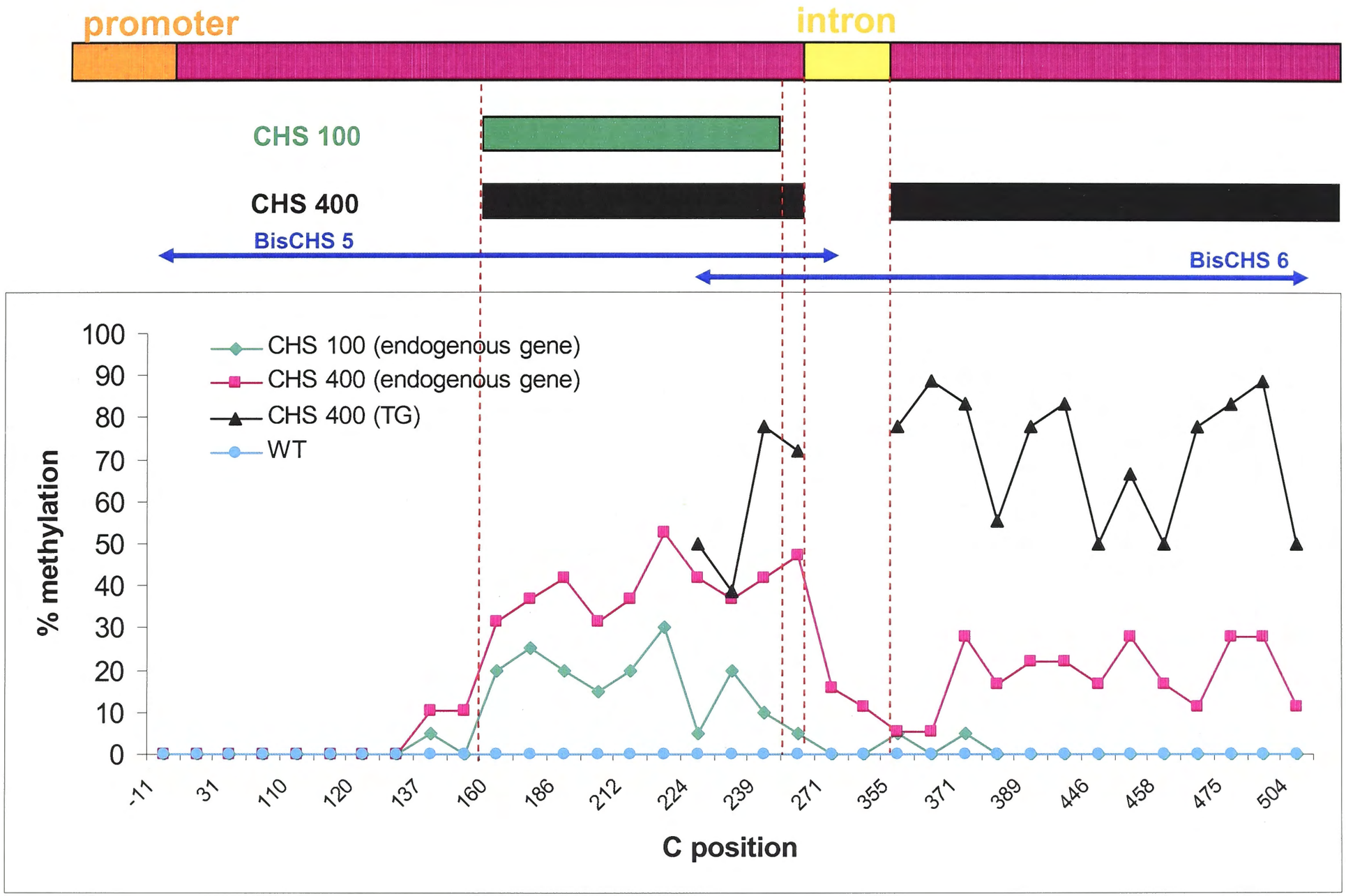

promoter
intron
CHS 100
CHS 400
BisCHS 5
BisCHS 6
CHS 100 (endogenous gene)
CHS 400 (endogenous gene)
CHS 400 (TG)
WT
% methylation
C position
100
90
80
70
60
50
40
30
20
10
0
-11
31
110
120
137
160
186
212
224
239
271
355
371
389
446
458
475
504

Fig. 4.33: Analysis of methylation of individual non-symmetric cytosine residues in plant lines carrying CHS100 or CHS400 hpRNA transgene.

Seed from plant lines CHS100 and CHS400 was germinated on MS medium containing Kanamycin. WT seed was germinated at the same time, but on MS medium that did not contain Kanamycin selection.

Two weeks after germination, leaf tissue was collected from at least five plants from each of the analysed plant lines. DNA was extracted, treated with sodium bisulfite and methylation analysed as described in section 4.2.11.

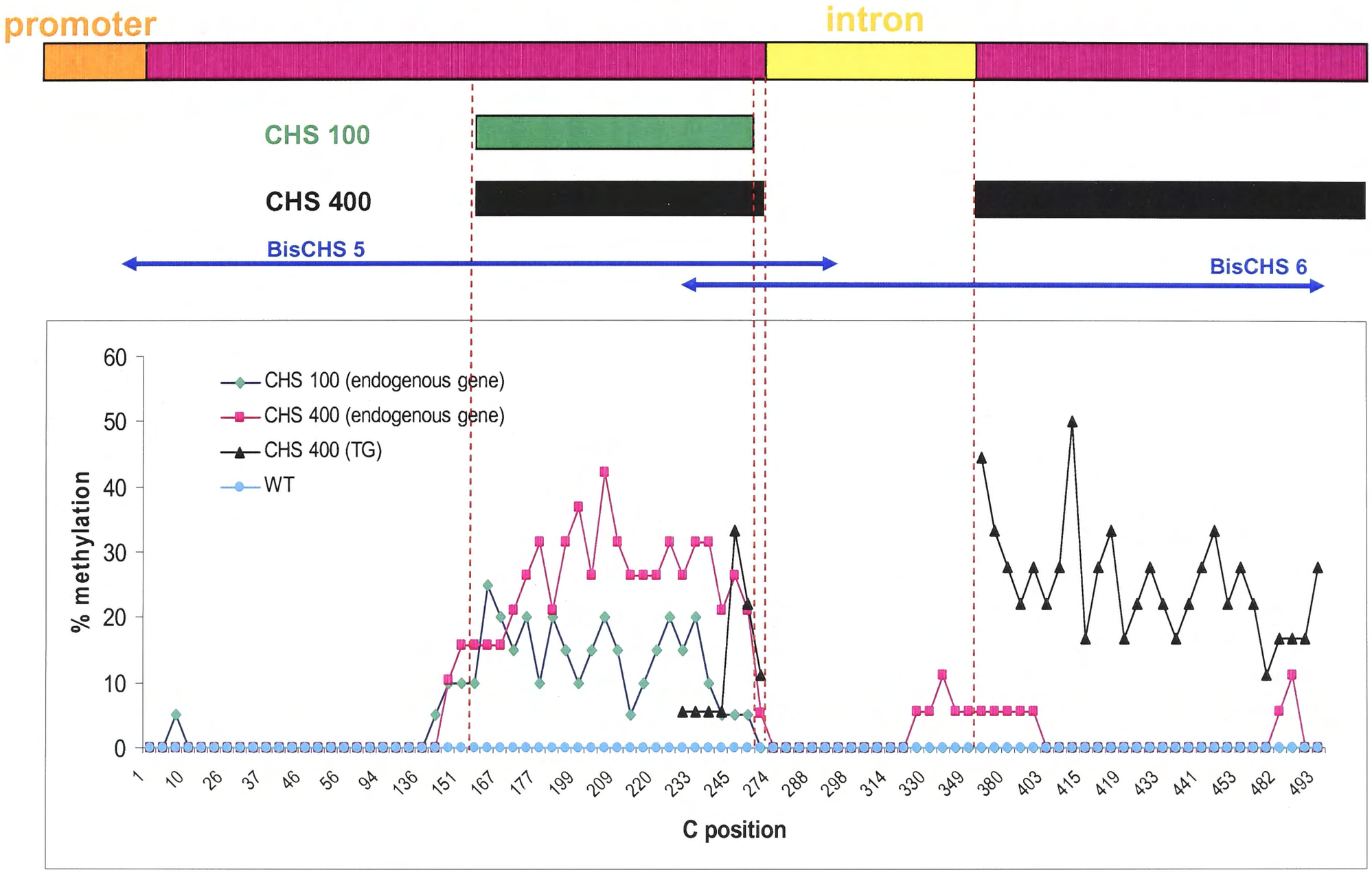
The pink bar represents the endogenous *CHS* gene. The orange section of this bar represents the promoter region while the yellow section represents the intronic region.

The green and black bars represent the CHS100 and CHS400 hpRNA transgenes, respectively. Bars representing transgenes are positioned parallel to the sections of the endogenous gene from which the transgene sequence was derived.

The blue lines with arrows represent the bisulfite PCR products used to analyse methylation patterns of the endogenous *CHS* sequences and the CHS400 hpRNA transgene. Methylation of endogenous *CHS* sequences was analysed using BisCHS5 and BisCHS6 products. Methylation of the CHS400 hpRNA transgene could only be analysed using the BisCHS6 product.

At least 20 sequences were obtained for each plant line. The percentage of methylation reflects the number of sequences (out of a total of 20) in which a particular cytosine residue was methylated.

The green line on the graph represents the methylation state of the endogenous *CHS* gene in a plant line that carries the CHS100 hpRNA transgene. The pink line on the graph represents the methylation state of the endogenous *CHS* gene in a plant line that carries the CHS400 hpRNA transgene while the black line represents the methylation state of the CHS400 hpRNA transgene in the same plant line. The gap in the black line appears because the CHS400 hpRNA transgene does not contain the intronic sequence.


promoter
intron
CHS 100
CHS 400
BisCHS 5
BisCHS 6
CHS 100 (endogenous gene)
CHS 400 (endogenous gene)
CHS 400 (TG)
WT
% methylation
0
10
20
30
40
50
60
1
10
26
37
46
56
94
136
151
167
177
199
209
220
233
245
274
288
298
314
330
349
380
403
415
419
433
441
453
482
493
C position

located in a CnG trinucleotide. Similarly, the intron of the *CHS* gene is not targeted by the CHS400 hpRNA construct but methylation spreads from the targeted 3' exon to the intronic cytosine at position 320. Interestingly, the spread in all three instances is 19-22 nucleotides. This is less extensive spreading than from the TGS-inducing hairpins (~100nts).

4.4 Discussion

This chapter describes the methylation patterns associated with TGS and PTGS. Analysis of cytosine methylation in the endogenous *CHS* gene targeted by a hpRNA transgene and the hpRNA transgene itself revealed that there was considerable variability in the level of cytosine methylation. Although the sample size was small, in terms of plant lines and hpRNA constructs, the data presented in this chapter suggest that:

- ~80% of symmetric cytosines (CG or CnG) located within the hpRNA transgenes were methylated
- 20-30% of non-symmetric cytosines located within the hpRNA transgenes were methylated.
- When the hpRNA transgene targeted the endogenous *CHS* promoter, the transgene and its endogenous target had similar levels of symmetric cytosine methylation. However, the targeted endogenous sequence had a higher level of non-symmetric cytosine methylation.
- Relative to the hpRNA transgene, methylation levels of the endogenous target were lower if the targeted region was transcribed.
- In the case of TGS, the shortest construct consistently produced the highest levels of methylation in the endogenous target.

- In the case of PTGS, the longer construct produced a slightly higher level of methylation in the endogenous target.
- Methylation levels were not uniform throughout the targeted area. In general, the 3' end of the targeted area had lower methylation levels.

  A similar pattern of methylation in loci targeted by RdDM has previously been reported. Stam *et al.* (1998) noticed that loci containing inverted repeats were densely methylated in the central section of the inverted repeat while the edges of these loci had lower methylation levels. Perhaps higher methylation levels in the central section of the targeted region could be due to the greater efficiency of production of siRNAs from the central region of the hairpin. Conversely, the 3' section of the hpRNA structure, which is closest to the intronic region of the hairpin construct, could be less efficiently processed and therefore generate a lesser amount of siRNAs that are able to guide methylation.
- Cytosine methylation was not confined to the targeted region.
  - The spread of methylation in TGS extended to cytosines that were up to 100bp removed from the targeted region.
  - In the case of PTGS, the spread of methylation extended to cytosines that were up to 25bp removed from the targeted region.

  It is possible that the difference in the extent of spread could be related to the levels of methylation observed in regions that are directly targeted by TGS and PTGS inducing hpRNA constructs. For example, ~ 80% of cytosines in regions directly targeted by the CHS3 constructs are methylated. In the case of PTGS, however, methylation levels of targeted regions are between 20 and 40%.

Perhaps the most interesting finding described in this chapter is that methylation is not confined to the original target sequence. Since cytosine methylation correlates with gene silencing, the spread of cytosine methylation associated with PTGS and TGS is likely to result in silencing of sequences that flank the original target.

A current model for the spread of PTGS proposes that the spread along the target molecule is most likely due to an amplification step mediated by the RdRP (Sijen *et al.* 2001a, Lipardi *et al.* 2001). According to this model, the spread of PTGS, and the accompanying spread of methylation, could affect sequences that are several hundred bases removed from the regions targeted by the PTGS inducing transgenes. Although this type of spread has been observed in *C. elegans*, *Drosophila* embryo extracts and in some cases of virus induced gene silencing (VIGS), it does not appear to apply to the endogenous plant genes. For example, the spread of PTGS is observed when sections of green fluorescent protein (GFP) are targeted by a virus-based silencing inducer (Vaistij *et al.* 2002). In contrast, no spread is observed when sections of endogenous plant genes, such as phytoene desaturase (PDS) or ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco), are targeted by a similar construct (Vaistij *et al.* 2002).

Previous studies have not thoroughly investigated or reported significant spread of silencing and methylation associated with TGS. As such, there is no current model that attempts to explain the spread of methylation observed in the experiments described in this chapter. It is appears unlikely that the RdRP-based model for the spread of PTGS can explain the spread of methylation observed in TGS. In particular, in the case of TGS there is no RNA template that could be used for RdRP-mediated amplification and spread.

However, if this type of mechanism were to explain the spread of TGS, it would require a polymerase able to use a siRNA primer and a DNA template to synthesise an RNA molecule. The process would be further complicated by the need to convert the single stranded RNA product into a double stranded one. Since the genetic and biochemical studies of proteins involved in TGS have not identified this type of polymerase it appears more likely that the spread of methylation associated with TGS is due to an alternative mechanism.

An alternative explanation for the spread of methylation is that the methyltransferases involved in *de novo* methylation may not confine their activity solely to the cytosine residues complementary to the siRNAs that guide the methyltransferase to the DNA target. The crystal structure of a prokaryotic methyltransferase suggests that the footprint of this enzyme is approximately 20bp (Chen *et al.* 2003). As eukaryotic methyltransferases are considerably larger than their prokaryote counterparts, these enzymes may be able to methylate cytosine residues that are up to 100bp further upstream from their primary target.

Another attractive possibility is that the spread of methylation may be the result of cooperative action of DNA methyltransferases, histone methyltransferases and perhaps additional proteins involved in the establishment of the inactive chromatin state (heterochromatin) (Fig. 4.34). siRNA-directed *de novo* methylation of cytosine residues in all sequence contexts appears to be mediated by domain re-arranged methyltransferases (DRMs) (Cao *et al.* 2003). It is likely that proteins containing the methyl-binding domains (MBDs) bind such methylated sequences and recruit histone methyltransferases such as KRYPTONITE (KYP) (Fujita *et al.* 2003, Fuks *et al.* 2003,). KYP-mediated methylation

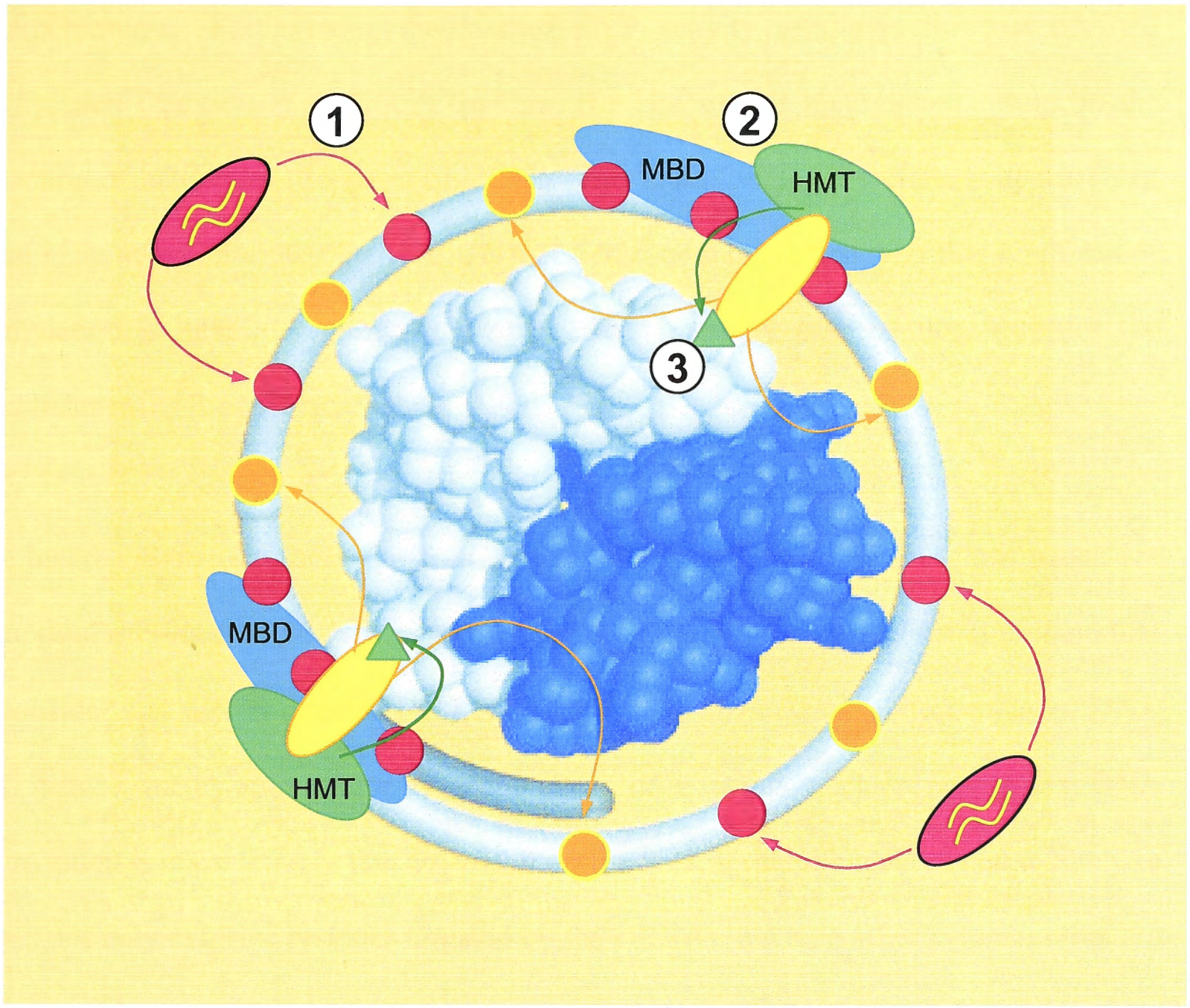


Figure 4.34: A model outlining the processes that result in the spread of cytosine methylation into the sequences that are not directly targeted by the hpRNA transgenes.

Nucleosome structure was adapted from Lewin (2003). In this structure H2A-H2B dimers are shown in blue while H3-H4 tetramer is shown in white.

① siRNA-guided *de novo* methylation (●) of cytosine residues in all sequence contexts is established by DRM methyltransferases.

② Methyl-binding proteins (MBD) are recruited to the methylated DNA sequences and they in turn recruit histone methyltransferases (HMT).

③ Methylation of H3K9 (▲) results in recruitment of DNA maintenance methyltransferases such as CMT3 and MET1(⬭).

Cross-talk between histone and DNA methylation is established resulting in reinforcement of both histone and DNA methylation.

Most cytosines within the loop of DNA wrapped around the histone octamer become methylated.

of histone H3 at the lysine 9 residue (H3K9Met) is associated with the establishment of heterochromatin. Furthermore, the loss of KYP activity results in the loss of CnG methylation (Jackson *et al.* 2002). In turn, the loss of CG methylation results in the reduction of H3K9Met (Soppe *et al.* 2002, Tariq *et al.* 2003). Therefore, it appears that there is an active interplay between DNA and histone methylation, and that one form of methylation is able to reinforce the other. This interplay may be necessary for the establishment of the heterochromatic state.

The heterochromatic state is characterised by tight packaging of nucleosomes which are composed of 146bp of DNA wrapped around the histone core. Therefore, it is important to consider that siRNA directed DNA methylation and subsequent reinforcement of DNA and histone methylation occur in the context of a whole nucleosome. When DNA methylation is considered in this context, it becomes clear that the methylation is likely to affect not only cytosine residues targeted by the siRNAs, but also other cytosines that are located within the 146bp of DNA wrapped around the histone core (Fig. 4.34). It is interesting to note that the spread of cytosine methylation described in this chapter is limited to approximately 100bp from the last directly targeted nucleotide. This distance of spread would be consistent with the need to reinforce methylation of DNA within a single nucleosome.

It is also interesting to re-consider in this context the difference in the levels and the spread of methylation observed in TGS and PTGS. As mentioned above, the spread of methylation in TGS affects cytosines that are up to 100bp removed from the targeted region while in PTGS the spread is limited to cytosines that are within 25bp from the targeted region. In TGS, siRNAs target methylation to non-transcribed regions where the

assembly of proteins involved in DNA and histone methylation can occur without being disrupted by transcriptional processes. In PTGS, however, methylation is targeted to the coding regions that are being continually transcribed. The transcription process is likely to disrupt the assembly of DNA and histone methyltransferases that are necessary for the reinforcement of methylation, the establishment of heterochromatin and more extensive spread of methylation. As such, in the case of PTGS, a large proportion of the methylation we observe may be a direct product of siRNA-targeted methyltransferases while in TGS higher levels of methylation may be due to continual reinforcement of the originally established siRNA-directed methylation.

Chapter 5

Summary of major findings and future directions

5.1 Major findings described in this thesis

This thesis investigated three different aspects of gene silencing, namely: the characteristics of cross protection and transgene mediated virus protection, the systemic spread of gene silencing and the spread of gene silencing along the target molecule. The major findings relating to these aspects of gene silencing are outlined below.

5.1.1 Characterisation of cross protection and transgene mediated virus protection

Experiments involving inoculations with PVY strains under several test conditions were used to compare and evaluate cross protection and transgene mediated protection.

- *In planta* expression of a suppressor of gene silencing, HC-Pro, had no effect on the efficiency of cross-protection. In contrast, *in planta* expression of HC-Pro rendered transgene mediated virus protection ineffective. These findings suggest that cross protection and transgene mediated protection may operate via two distinct branches of the gene silencing pathway.
- Both S+AS and hpRNA transgenes provided very efficient and robust protection against the PVY strain from which the transgene was derived and a suite of related viral strains. The minimum continuous sequence identity between the transgene and the challenging viral strain was 20bp.

5.1.2 Systemic spread of gene silencing

A series of grafting experiments were used to investigate the effect of transgene structure, grafting method, plant age and the inducer/target homology on the systemic spread of PTGS.

- hpRNA transgenes were able to generate and transmit a silencing signal while the S+AS transgenes were not able to do so.
- The systemic spread of the silencing signal was unidirectional – from the rootstock to the scion.
- The ability of scion tissue to process the signal and establish silencing was dependent on the grafting method and the age of the tissue that received the signal.
- The silencing signal could only be used to establish effective silencing in the recipient tissue if the transgene from which the signal was derived and its target shared a 100% sequence identity.

5.1.3 Spread of silencing along the target molecule

DNA methylation patterns were analysed to determine whether methylation, and therefore gene silencing, spreads into sequences flanking the directly targeted area.

- This study is the first to investigate, in detail, the spread of methylation in a TGS system. I found that, in a TGS experimental system, methylation spread to the areas that were approximately 100bp outside sections that were directly targeted by the hpRNA transgenes.
- Previous studies have argued that the spread of PTGS can be extensive, affecting sequences that are hundreds of bases far from the directly targeted area. However, in this study I found that the spread of methylation, and

therefore PTGS, was limited to an area that was approximately 20bp outside sections that were directly targeted by the hpRNA transgenes.

5.2 Raising questions and indicating future directions

5.2.1 Do cross protection and transgene mediated virus protection operate via two distinct branches of the gene silencing pathway?

In Chapter 2 I have shown that cross protection and transgene mediated virus protection offer a similar breadth of protection against a suite of divergent PVY strains. However, cross protection was not affected by a suppressor of gene silencing, HC-Pro, while the same protein rendered transgene mediated protection ineffective.

I outlined two possibilities that could account for this observation:

1. *In planta* expressed HC-Pro, derived from tobacco etch virus (TEV), could have been cross-silenced during the primary inoculation with PVY. Consequently, in planta expressed HC-Pro could not interfere with cross protection that occurred during the secondary inoculation with a necrotic PVY strain.

2. Alternatively, cross-protection and transgene mediated protection could operate via two distinct gene silencing pathway. In this case, HC-Pro, and perhaps other viral suppressors of gene silencing, could have a different impact on these two modes of viral protection.

Although it is very unlikely that *in planta* expressed HC-Pro was cross-silenced (see section 2.4) this hypothesis cannot be rejected without further experimental evidence. The effect of HC-Pro on cross protection could be investigated using viruses that do not belong to the *Potiviridae* family and therefore do not carry HC-Pro sequences that

have a potential to cross-silence the *in planta* expressed HC-Pro. Alternatively, Northern blotting or reverse transcriptase PCR (RT-PCR) could be used to analyse expression of *in planta* expressed HC-Pro during the primary infection with PVY. Although both TEV (from which *in planta* expressed HC-Pro was derived) and PVY are *Potyviridae*, sequence comparisons indicate that their HC-Pro sequences are sufficiently different to allow for this type of analysis. Detection of a significant amount of TEV-HC-Pro RNA would indicate that *in planta* expression of this gene has not been silenced and that the TEV-HC-Pro protein is probably synthesised, but not able to interfere with cross-protection. If this is indeed the case, then further experimental work should aim to further characterise the mechanism of cross protection.

An interesting approach to the investigation of the mechanisms of cross protection would be to test the efficiency of cross-protection in the presence of various other suppressors of gene silencing. This approach is already being used to characterise branches of the silencing pathway specific to sense transgenes, amplicon constructs, hpRNA transgenes and micro RNAs (miRNAs). For example, it is known that p69, a suppressor of gene silencing encoded by turnip yellow mosaic virus, inhibits sense-mediated and amplicon mediated silencing. However, p69 does not affect hpRNA mediated silencing or miRNA pathways. These findings indicated that p69 interferes with the formation of dsRNA that occurs in the sense and amplicon silencing pathways before they merge with hpRNA and miRNA silencing pathways (Chen *et al.* 2004). In contrast, p19, a suppressor of gene silencing encoded by potato virus X, affects all branches of the silencing pathway. p19 is known to suppress silencing by sequestering and binding both siRNAs and miRNAs, indicating that all branches of the

silencing pathway converge at this point (Silhavy *et al.* 2002, Papp *et al.* 2003, Silhavy & Burgyan 2004).

There have been attempts to use HC-Pro to further characterise different branches of the gene silencing pathway, but these have not always yielded clear answers. It is now accepted that HC-Pro allows the processing of double stranded miRNA precursors but blocks the miRNA guided cleavage of target mRNA molecules (Kasschau *et al.* 2003, Mallory *et al.* 2003, Dunoyer *et al.* 2004). The effect of HC-Pro on other branches of the silencing pathway is less clear. Johansen & Carrington (2001) investigated the effect of HC-Pro on PTGS in a system where PTGS of green fluorescent protein (GFP) is induced by agro-infiltration of single stranded GFP (ssGFP) or hpRNA GFP transgenes. In this system HC-Pro completely suppressed ssGFP induced silencing, but only reduced the efficiency of hpRNA-mediated silencing. Furthermore, HC-Pro did not prevent the formation of siRNAs, which appeared to be consistent with its effect on the miRNA silencing pathway. On the other hand, HC-Pro was able to effectively suppress PTGS mediated by hpRNA transgenes integrated into the plant genome. However, in this case, HC-Pro appeared to decrease the efficiency of dsRNA processing resulting in reduction, but not complete elimination, of siRNAs. (Mallory *et al.* 2003, Dunoyer *et al.* 2004).

Although studies analysing the effects of HC-Pro on different branches of the silencing pathway have not yielded consistent results, they have generated enough data to allow us to develop a model proposing a possible mode of action for HC-Pro (Silhavy & Burgyan 2004). According to this model, HC-Pro is likely to block the activity of RISC. However, RISC operates at the last step of the silencing pathway

where all branches of gene silencing pathways should merge. If this were the case, HC-Pro would be expected to have the same effect on all of these branches. Alternatively, HC-Pro could target a particular protein component of RISC that may not be involved in all branches of the silencing pathway. If this is the case, then cross protection may involve a yet unidentified protein that cannot be inhibited by HC-Pro, but can become a component of RISC. Identification of such a protein would add another chapter to the story about the continuous efforts of plants and viruses to evolve ways of out-smarting each other.

5.2.2 When do we see differences between S+AS and hpRNA transgenes – and why?

Although both S+AS and hpRNA transgenes were equally efficient at providing protection against a suite of PVY strains further characterisation of these transgenes revealed that they may operate via two partly distinct branches of the silencing pathway. The major differences between these two transgenes were:

1. Although very young plants carrying hpRNA or S+AS transgenes accumulated some virus, the recovery of hpRNA plants was very rapid while S+AS plants recovered more slowly. The rate of their recovery was dependant on the number of sequence identity blocks the S+AS transgenes shared with the challenging virus. The greater the number of shared sequence identity blocks the faster the recovery.
2. The hpRNA transgene produced a large amount of siRNAs prior to viral infection, while only a small amount of siRNAs was detected in the plants carrying S+AS transgene.

3. Tissues carrying the hpRNA transgene produced a graft transmissible silencing signal that was able to induce silencing in the scion tissues while tissues carrying the S+AS tranagenes were not able to produce such a signal.
4. hpRNA transgenes were able to induce the silencing of viral genes, transgenes (GUS) and endogenous genes (CHS). Although the ability of S+AS transgene to induce silencing of an endogenous plant gene was not tested, the S+AS transgenes were not able to mediate GUS silencing.

Interestingly, when plants expressing S+AS GUS transgenes were inoculated with PVY, approximately 10% of the inoculated plants displayed GUS silencing. It is not clear why the presence of PVY induced GUS silencing. One possibility is that the presence of a virus could up-regulate the detection system used by the plant to identify sequences with the potential to form double stranded RNA molecules. At least in some PVY infected plants, GUS silencing appears to be mosaic suggesting that silencing may only be activated in cells that contain the virus. It would be interesting to determine whether this is indeed the case. However, because PVY symptoms are not always obvious, PVY-infected and virus-free cells cannot be easily identified and examined for GUS expression. This problem could be overcome by infecting tobacco plants with cucumber mosaic virus (CMV), which causes yellowing of infected areas and leaves green islands in the areas where the virus is not present. The relationship between the viral presence and onset of GUS silencing could then be investigated by staining yellow and green areas for GUS expression. This type of experiment would also help determine whether the ability to induce S+AS mediated silencing is unique to PVY.

It would also be interesting to investigate whether a specific viral protein is sufficient to induce S+AS mediated silencing. For example, a construct encoding a viral RdRP could be super-transformed into plants carrying an active GUS construct and S+AS GUS transgenes. From previous observations we would expect a relatively high GUS activity in plants carrying only the GUS construct and S+AS GUS transgenes. However, loss of GUS activity in transformants carrying the viral RdRP, GUS construct and S+AS GUS would indicate that viral RdRP is the viral factor responsible for the initiation of S+AS mediated silencing. If none of the individually expressed viral proteins was able to induce S+AS mediated silencing, then the up-regulation of plant's dsRNA detection system, in response to a viral infection, may account for the effectiveness of the S+AS transgenes in the presence of PVY.

The apparent inability of S+AS transgenes to initiate silencing in the absence of a virus may also account for the inability of these transgenes to induce systemic silencing as tested by the grafting experiments. In these experiments AS or WT scions susceptible to PVY were grafted onto hpRNA or S+AS rootstocks that were known to be resistant to PVY. PVY challenge and subsequent detection of viral infection in the scion was used to test for the spread of silencing from S+AS or hpRNA rootstocks into the scions. Resistance of the scion tissues to PVY was indicative of the systemic spread of the silencing.

It is important to note that neither hpRNA nor S+AS rootstocks were inoculated with PVY prior to grafting. This may have been of particular significance to S+AS rootstocks because, unless a virus is present, S+AS transgenes do not generate significant levels of siRNAs and may not be able to initiate silencing. As such,

silencing and the subsequent production of the silencing signal may not have been initiated in non-inoculated S+AS rootstocks. It would be interesting to repeat the grafting experiments described in this thesis using pre-inoculated S+AS rootstocks. It is likely that pre-inoculated S+AS rootstocks will be able to produce and transmit a silencing signal. Testing whether pre-inoculation of the hpRNA rootstocks upregulates the production of the silencing signal and promotes its transmission could extend these studies even further.

5.2.3 What is the minimum required sequence identity between the virus and the transgene?

Previous studies have analysed the minimum sequence identity, between an siRNA and its target RNA, that is required for effective PTGS. Studies using *in vitro* experimental systems have found that the minimum required sequence identity ranges from 19 to 21 nucleotides while the studies conducted *in vivo* suggest the required sequence identity ranges from 21 to 23 nucleotides (Boutla *et al.* 2001, Elbashir *et al.* 2001c, Thomas *et al.* 2001, Martinez *et al.* 2002). *In vivo* experiments described in this thesis indicate that effective PTGS could be achieved even when the S+AS transgenes and a challenging virus share only 20nt of continuous sequence identity. It should be mentioned, however, that most of the previous studies used either synthetic siRNAs or small fragments of the silencing inducer integrated within an unrelated sequence. In contrast, in the experimental I used, the 20nt sequence identity blocks were embedded into a viral sequence that had an overall homology with the S+AS transgene of 84%.

Experiments described in section 2.3.4 and a finding that an overall homology of 89% between a transgene and a PVY strain does not result in PTGS (Maki-Valkama *et al.* 2000) indicate that a 20nt sequence identity block, rather than the background

homology, allows the transgenes to effectively induce PTGS of a viral sequence. However, it would still be beneficial to develop an experimental system that could test whether a virus carrying only a 20nt sequence identity block and no other sequences that share homology with the transgene, could be effectively targeted by S+AS or hpRNA transgenes. Such a system could be developed by integrating small blocks of PVY sequence into the genome of an unrelated virus. For example, cassettes of PVY sequence could be introduced into the genome of tobacco mosaic virus (TMV) that already carries GFP sequence as a marker of TMV infection. This system could be used not only to verify the findings described in this thesis, but also to investigate further the impact of sequence identity on the dynamics of PTGS. For example, PVY cassettes of different lengths and perhaps with varied mismatch patterns could be introduced into TMV-GFP. Successful infection by these recombinant viruses could be scored by GFP expression. These viruses could be used to answer the following questions:

1. Do plant tissues carrying the S+AS or hpRNA transgene eliminate the virus at the point of entry, or are a few cells in the inoculated leaf infected during a struggle between the virus' offence and the plant's defence?
2. Is the dynamics of virus elimination different in plants carrying S+AS or hpRNA transgenes?
3. Is a recombinant virus that shares a sequence identity of only 20nt, or less, with S+AS or hpRNA transgenes, eliminated immediately upon inoculation, or does it succeed in infecting some sections of the leaf?

4. What is the impact of mismatches within a sequence identity block on the ability of S+AS and hpRNA transgenes to eliminate the virus?

5.2.4 How does the spread of silencing travel along the target molecule?

As the spread of TGS along the target molecule has not been extensively investigated a model that could account for this phenomenon has not yet been developed. In contrast, the spread of PTGS has been investigated in several experimental systems and a model accounting for this spread has been extensively discussed (Lipardi *et al.* 2001, Sijen *et al.* 2001a, Vaistij *et al.* 2002). According to this model, the spread of PTGS along the target molecule is due to the RdRP-mediated amplification that uses a target RNA molecule as a template. Support for this model has come from the studies that found that the spread of PTGS was abolished in plant lines with mutations in plant RdRP (Vaistij *et al.* 2002). An amplification step could also account for the ability of S+AS transgene to mediate silencing of viral sequences that share only two 20bp sequence identity blocks with this transgene. In this case, siRNAs derived from the S+AS transgene could act as primers for amplification from the viral RNA. As a result of this amplification step, dsRNA and many siRNAs with complete identity to the virus could be synthesised and used to guide cleavage of viral RNA.

Ideally the amplification hypothesis would be tested using S+AS plants infected with an appropriate PYV strain. However, there are several complications that make this experimental system unsuitable for investigation of spread of PTGS. Firstly, if siRNAs derived from S+AS transgenes were to be used as primers for the amplification of viral RNA, then the products would be dsRNA and siRNAs derived from viral sequences. However, during PVY infection siRNAs are also generated from the viral genome

itself. Therefore it would not be possible to determine whether virus-derived siRNAs were the product of RdRP mediated amplification or a by-product of viral infection. Furthermore, even if there was a way to determine the origin of siRNAs, we would not be able to determine whether the amplification was catalysed by a plant RdRP or viral RdRP. This distinction is important because amplification by a plant RdRP might indicate that this step is a part of an endogenous silencing pathway, while amplification by a viral RdRP could be specific to the virus induced gene silencing pathway.

Because virus infected S+AS plants were not a suitable system for investigation of the spread of gene silencing I developed a new system that allowed for an easy appraisal of silencing efficiency and the analysis of spread of both TGS and PTGS. In this system TGS or PTGS of the endogenous CHS gene was induced by hpRNA constructs derived from either the promoter or coding CHS sequence. Because siRNAs guide DNA methylation of homologous sequences, an increase in DNA methylation levels within sequences flanking the primary target can be indicative of the spread of gene silencing. Experiments described in Chapter 4 indicate that the spread of methylation in TGS affects cytosines that are up to 100bp removed from the targeted region while in PTGS the spread is limited to the cytosines that are within 25bp from the targeted region.

As discussed in section 4.4.5, RdRP mediated amplification is not likely to account for the spread of methylation observed in TGS. Furthermore, RdRP-mediated spread is likely to be much more extensive than the 25bp spread observed in PTGS. As such, RdRP-mediated amplification does not appear to account for the spread observed in

silencing induced by hpRNA transgenes. It is still possible that more extensive spread, perhaps mediated by RdRP, could occur in S+AS induced silencing. This could be another important point of distinction between these two transgenes and is a topic worthy of further investigation.

Several possible scenarios that could lead to the spread of methylation described in this thesis were discussed in section 4.4.5. Perhaps the most appealing possibility is that the spread of methylation is due to the cross talk between proteins involved in DNA methylation and histone methylation. According to this hypothesis the first layer of methylation is established by *de-novo* DNA methyltransferases that are guided by siRNAs derived from the hpRNA transgene. This methylation would be limited to the primary target sequences. This type of methylation could lead to the recruitment of histone methyltransferases and maintenance DNA methyltransferases (Fig. 4.34). During this process, methylation would be likely to affect not only cytosine residues targeted by the siRNAs, but also other cytosines that are located within the 146bp of DNA wrapped around the histone core (Fig. 4.34). This hypothesis could be tested by mapping the position of nucleosomes in the *A. thaliana* plant lines that have been described in this thesis (see chapter 4). If the nucleosomes are positioned over the sequences affected by the spread of methylation, then this would support the hypothesis that the spread of DNA methylation occurs because cells respond to the siRNA guided DNA methylation by modifying both histones and the entire loop of DNA that is wrapped around the histone core.

5.2.5 Does the spread of silencing along the target molecule travel in both directions?

Experiments described in this thesis examined, in detail, the spread of silencing along the target molecule in the 5' direction. However, only limited information was obtained about the spread of silencing in the 3' direction. In the case of TGS the CHS constructs targeted the endogenous CHS promoter from the nucleotide positioned at -11, therefore not leaving enough promoter sequence for the analysis of the 3' spread. In the case of PTGS, very low level of methylation was detected in sequences located 3' from the section targeted by the CHS100 hpRNA transgene. However, because only one plant line was analysed, this phenomenon would need to be investigated further.

Although the 3' spread of TGS has not been extensively studied, the available data suggest that the 3' spread is not as extensive as the 5' spread. For example, Aufsatz *et al.* (2002) analysed approximately 60bp of sequence located 3' from the targeted region, but found that only 2 out of 130 analysed cytosines were methylated. Furthermore, the two methylated cytosines were located within 15bp from the targeted region. Similarly, analysis of methylation patterns in plant transposons revealed that the transposons themselves and the 5' flanking sequence were methylated but no spread of methylation into the 3' flanking sequence was identified (Dr Peter Waterhouse and Dr Adriana Fusaro *pers. comm.*).

The 3' spread of PTGS has been studied before, but the findings have not been consistent. For example, 3' spread of PTGS has been detected in experimental systems involving *Drosophila* embryo extracts and virus inoculated plants. However, no 3' spread was detected in a *C. elegans* system (Lipardi *et al.* 2001, Sijen *et al.* 2001a, Vaistij *et al.* 2002,). Similarly, detailed analysis of methylation patterns associated

with satellite RNA-directed methylation did not reveal any spread of methylation into sequences positioned 3' from the targeted region (Wang *et al.* 2001).

Future studies could extend the work described in this thesis by investigating the spread of silencing in the 3' direction. In the case of TGS this will probably involve generating new CHS constructs that target promoter sections that are at least 200bp removed from the start of transcription. The 3' spread of methylation, and TGS, in plant lines carrying such constructs could be analysed by the bisulfite sequencing approach as described in this thesis. The spread of PTGS in the 3' direction could be investigated using the existing plant line that carries the CHS400 construct. However, because this line has low methylation levels in the 3' end of the targeted section it may be difficult to detect the spread into the 3' flanking sequences. It is likely that the spread of PTGS could be better investigated in new plant lines carrying either the existing CHS400 construct or a newly designed, but perhaps shorter, construct.

Appendix 1

Figure A1.1: Sequence comparison of PVY-D derived transgene and 18S1 PVY strain.

```
                1                                                          60
PVY-D                                        GTC G--AACGTGA AGGACATACC AGCACAGGAG
18S1    GAACTAAGGC AAACTGGGCC AGCTGTGGAA GTTAACGTGA AGGACATACC AAAACAGGAG
Consensus ..........  ..........  .......Gaa G..AACGTGA AGGACATACC AaaACAGGAG

               61                                                         120
PVY-D   GTGGAGCACG AAGCTAAATC GCTCATGAGA GGCTTGAGAG ACTTTAACCC AATTGCCCAA
18S1    GTAGAGCATG AAGCTAAATC GCTCATGAGG GGTTTAAGAG ATTTCAATCC AATTGCCCAA
Consensus GTaGAGCAcG AAGCTAAATC GCTCATGAGa GGcTTaAGAG AcTTcAAcCC AATTGCCCAA

              121                                                         180
PVY-D   ACAGTTTGTA GGCTGAAAGT ATCTGTTGAA TATGGGACAT CAGAGATGTA CGGTTTTGGA
18S1    ACGGTTTGTA GGTTGAAAGT ATCCGTTGAG TTTGGAACGT CAGAGCTATA TGGTGTTGGA
Consensus ACaGTTTGTA GGcTGAAAGT ATCcGTTGAa TaTGGaACaT CAGAGaTaTA cGGTgTTGGA

              181                                                         240
PVY-D   TTTGGAGCAT ACATAATAGC GAACCACCAT TTGTTCAGGA GTTATAATGG TTCCATGGAG
18S1    TTTGGGGCAT ATATTATAGC GAACCACCAT TTGTTTAGGA GTTACAATGG TTCAATGGAA
Consensus TTTGGaGCAT AcATaATAGC GAACCACCAT TTGTTcAGGA GTTAcAATGG TTCaATGGAa

              241                                                         300
PVY-D   GTGCGATCTA TGCACGGTAC ATTCAGGGTG AAGAATCTAC GCAGTTTGAG CGTTCTGCCA
18S1    GTGCGATCTA TGCATGGCAC GTTCAGGGTG AAGAACCTAC ACAGTTTGAG TGTTTTGCCA
Consensus GTGCGATCTA TGCAcGGcAC aTTCAGGGTG AAGAAcCTAC aCAGTTTGAG cGTTcTGCCA

              301                                                         360
PVY-D   ATTAAAGGTA GGGATATCAT CCTCATCAAA ATGCCGAAAG ATTTCCCTGT CTTTCCACAG
18S1    ATTAAGGGTA GGGACATCAT CATCATCAAA ATGCCAAAGG ATTTCCCTGT CTTTCCACAG
Consensus ATTAAaGGTA GGGAcATCAT CaTCATCAAA ATGCCaAAaG ATTTCCCTGT CTTTCCACAG

              361                                                         420
PVY-D   AAATTGCATT TCCGAGCTCC AACACAGAAT GAAAGAGTTT GTTTAGTTGG AACCAACTTT
18S1    AAGCTACGTT TCCGCGCTCC AACACAGAAT GAAAGAATTT GTCTAGTCGG GACAAACTTC
Consensus AAacTaCaTT TCCGaGCTCC AACACAGAAT GAAAGAaTTT GTcTAGTcGG aACaAACTTc

              421                                                         480
PVY-D   CAGGAGAAGT ATGCATCGTC GATCATCACA GAGACAAGCA CCACTTACAA TATACCGGGC
18S1    CAAGAGAAAT ATGCATCTTC AATCATCACA GAAACTAGCA CTACTTACAA TGTGCCGGGT
Consensus CAaGAGAAaT ATGCATCgTC aATCATCACA GAaACaAGCA CcACTTACAA TaTaCCGGGc

              481                                                         540
PVY-D   AGCACATTCT GGAAGCATTG GATTGAAACA GATAATGGAC ATTGTGGACT ACCAGTGGTG
18S1    AGCACTTTTT GGAAGCACTG GATTGAGACG GATGATGGAC ATTGTGGATT GCCAGTAGTG
Consensus AGCACaTTcT GGAAGCAcTG GATTGAaACa GATaATGGAC ATTGTGGAcT aCCAGTaGTG

              541                                                         600
PVY-D   AGTACCACCG ATGGATGTCT AGTCGGAATC CACAGTTTGG CAAACAACAG ACACACCACG
18S1    AGTACTGCCG ATGGATGTCT GGTTGGAATA CATAGCTTGA CAAATAATGC GCAGTCCACG
Consensus AGTACcaCCG ATGGATGTCT aGTcGGAATa CAcAGcTTGa CAAAcAAcac aCAcaCCACG

              601                                                         660
PVY-D   AACTACTACT CAGCCTTCGA TGAAGATTTT GAAAGCAAGT ATCTCCGAAC CAATGAGCAC
18S1    AGCTACTACT CAGCCTTTGA TGAAGATTTC GAGAGCAAAT ATCTCCGAAC TAATGAGCAT
Consensus AaCTACTACT CAGCCTTcGA TGAAGATTTc GAaAGCAAaT ATCTCCGAAC cAATGAGCAc

              661                                                         720
PVY-D   AATGAATGGG TCAAGTCTTG GATTTATAAT CCAGACACAG TGTTGTGGGG CCCGTTGAAA
18S1    AATGAATGGA TTAAATCCTG GGTCTATAAT CCAGATACAG TGCTGTGGGG TCCTCTAAAA
Consensus AATGAATGGa TcAAaTCcTG GaTcTATAAT CCAGAcACAG TGcTGTGGGG cCCgcTaAAA

              721                                                         780
PVY-D   CTTAAAGACA GCACTCCCAA AGGATTATTC AAGACAACAA A
18S1    CTCAAGGAAA GCACTCCTAA AGGATTGTTT AAAACAACTA AGCTTGTGCA GGATTTGATA
Consensus CTcAAaGAaA GCACTCCcAA AGGATTaTTc AAaACAACaA A.........  ..........
```

Figure A1.2: Sequence comparison of PVY-D derived transgene and 431S1 PVY strain.

```
                1                                                        60
PVY-D                                                GTCGAACGTG AAGGACATAC CAGCACAGGA
431S    GAACTAAGGC AAACTGGGCC AGCTGTGGAA GTTGAT-GTG AAGGACATAC CAAAACAGGA
Consensus ..........  ..........  .......... GTcGAa.GTG AAGGACATAC CAaaACAGGA

               61                                                       120
PVY-D   GGTGGAGCAC GAAGCTAAAT CGCTCATGAG AGGCTTGAGA GACTTTAACC CAATTGCCCA
431S    GGTAGAGCAT GAAGCTAAAT CGCTCATGAG AGGTTTAAGA GATTTCAATC CAATTGCCCA
Consensus GGTaGAGCAc GAAGCTAAAT CGCTCATGAG AGGcTTaAGA GAcTTcAAcC CAATTGCCCA

              121                                                       180
PVY-D   AACAGTTTGT AGGCTGAAAG TATCTGTTGA ATATGGGACA TCAGAGATGT ACGGTTTGG
431S    AACAGTTTGT AGGCTGAAAG TCTCTGTTGA GTTTGGAACG TCAGAGTTAT ATGGTATTGG
Consensus AACAGTTTGT AGGCTGAAAG TaTCTGTTGA aTaTGGaACa TCAGAGaTaT AcGGTaTTGG

              181                                                       240
PVY-D   ATTTGGAGCA TACATAATAG CGAACCACCA TTTGTTCAGG AGTTATAATG GTTCCATGGA
431S    GTTTGGGGCA TACATTATAG CAAACCACCA CTTGTTTAAG AGCTATAATG GTTCAATGGA
Consensus aTTTGGaGCA TACATaATAG CaAACCACCA cTTGTTcAaG AGcTATAATG GTTCaATGGA

              241                                                       300
PVY-D   GGTGCGATCT ATGCACGGTA CATTCAGGGT GAAGAATCTA CGCAGTTTGA GCGTTCTGCC
431S    AGTACGATCT ATGCATGGTA CATTCAGGGT GAAGAACCTA CACAGTTTAA GTGTCTTACC
Consensus aGTaCGATCT ATGCAcGGTA CATTCAGGGT GAAGAAcCTA CaCAGTTTaA GcGTccTaCC

              301                                                       360
PVY-D   AATTAAAGGT AGGGATATCA TCCTCATCAA AATGCCGAAA GATTTCCCTG TCTTTCCACA
431S    AATTAAGGGT AGGGACATCA TCCTCATCAA AATGCCAAAG GACTTNCCTG TTTTNCCACA
Consensus AATTAAaGGT AGGGAcATCA TCCTCATCAA AATGCCaAAa GAcTTnCCTG TcTTnCCACA

              361                                                       420
PVY-D   GAAATTGCAT TTCCGAGCTC CAACACAGAA TGAAAGAGTT TGTTTAGTTG GAACCAACTT
431S    GAAGCTACGT TTCCGAGCTC CAACACAGAA TGAAAGAATT TGTCTAGTCG GAACAAACTT
Consensus GAAacTaCaT TTCCGAGCTC CAACACAGAA TGAAAGAaTT TGTcTAGTcG GAACaAACTT

              421                                                       480
PVY-D   TCAGGAGAAG TATGCATCGT CGATCATCAC AGAGACAAGC ACCACTTACA ATATACCGGG
431S    TCAGGAGAAG TATGCNTCTT CAATTATTAC AGAAACTAGC ACCACTTACA ACGTGCCGGG
Consensus TCAGGAGAAG TATGCaTCgT CaATcATcAC AGAaACaAGC ACCACTTACA AcaTaCCGGG

              481                                                       540
PVY-D   CAGCACATTC TGGAAGCATT GGATTGAAAC AGATAATGGA CATTGTGGAC TACCAGTGGT
431S    TAGTACTTTT TG-AAGCACT GGATTGAGAC GGACGATGGG CATTGTGGAT TACCAGTAGT
Consensus cAGcACaTTc TG.AAGCAcT GGATTGAaAC aGAcaATGGa CATTGTGGAc TACCAGTaGT

              541                                                       600
PVY-D   GAGTACCACC GATGGATGTC TAGTCGGAAT CCACAGTTTG GCAAACAACA GACACACCAC
431S    GAGTACTGCT GATGGATGTC TACTTGGAAT ACATAGTTTG GCAAACAATG CGCAGTCCAC
Consensus GAGTACcaCc GATGGATGTC TAcTcGGAAT aCAcAGTTTG GCAAACAAca caCAcaCCAC

              601                                                       660
PVY-D   GAACTACTAC TCAGCCTTCG ATGAAGATTT TGAAAGCAAG TATCTCCGAA CCAATGAGCA
431S    GAACTACTAC TCAGCCTTTG ATGAAGATTT CGAGAGCAAA TATCTCCGAA CTAATGAGCA
Consensus GAACTACTAC TCAGCCTTcG ATGAAGATTT cGAaAGCAAa TATCTCCGAA CcAATGAGCA

              661                                                       720
PVY-D   CAATGAATGG GTCAAGTCTT GGATTTATAA TCCAGACACA GTGTTGTGGG GCCCGTTGAA
431S    TAATGAATGG ATTAAATCCT GGGTCTATAA TCCGGATACA GTGCTGTGGG GCCCGCTAAA
Consensus cAATGAATGG aTcAAaTCcT GGaTcTATAA TCCaGAcACA GTGcTGTGGG GCCCGcTaAA

              721                                                       780
PVY-D   ACTTAAAGAC AGCACTCCCA AAGGATTATT CAAGACAACA AA
431S    GCTTAAAGAA AGCACTCCCA AAGGGTTGTT TAAAACAACT AAGCTTGTGC AGGATTTGGC
Consensus aCTTAAAGAa AGCACTCCCA AAGGaTTaTT cAAaACAACa AA........ ..........
```

Figure A1.3: Sequence comparison of PVY-D derived transgene and 120F PVY strain.

```
                1                                                          60
PVY-D                                        GTCGA A-----CGTG AAGGACATAC CAGCACAGGA
120F    TGAACTAAGG CAAACTGGGC CAGCTGTGGA AGTTTTTGTG AAGGACATAC CAAAACAGGA
Consensus ..........  ..........  .....GTcGA A.....cGTG AAGGACATAC CAaaACAGGA

               61                                                         120
PVY-D   GGTGGAGCAC GAAGCTAAAT CGCTCATGAG AGGCTTGAGA GACTTTAACC CAATTGCCCA
120F    GGGGGAACAT GAAGCTAAAT CGCTCATGAG AGGCTTAAGA GATTTTAATC CAATTGCCCA
Consensus GGgGGAaCAc GAAGCTAAAT CGCTCATGAG AGGCTTaAGA GAcTTTAAcC CAATTGCCCA

              121                                                         180
PVY-D   AACAGTTTGT AGGCTGAAAG TATCTGTTGA ATATGGGACA TCAGAGATGT ACGGTTTTGG
120F    AACAGTTTGT AGGCTGAAAG TGTCTGTTGA GTTTGGAACG TCAGAGTTAT ATGGTATTGG
Consensus AACAGTTTGT AGGCTGAAAG TaTCTGTTGA aTaTGGaACa TCAGAGaTaT AcGGTaTTGG

              181                                                         240
PVY-D   ATTTGGAGCA TACATAATAG CGAACCACCA TTTGTTCAGG AGTTATAATG GTTCCATGGA
120F    GTTTGGAGCA TACATTATAG CAAACCACCA CTTGTTCAAG AGCTACAATG GTTCAATGGA
Consensus aTTTGGAGCA TACATaATAG CaAACCACCA cTTGTTCAaG AGcTAcAATG GTTCaATGGA

              241                                                         300
PVY-D   GGTGCGATCT ATGCACGGTA CATTCAGGGT GAAGAATCTA CGCAGTTTGA GCGTTCTGCC
120F    AGTGCGATCT ATGCATGGTA CATTCAGGGT TAAGAACCTA CACAGTTTAA GTGTCTTGCC
Consensus aGTGCGATCT ATGCAcGGTA CATTCAGGGT gAAGAAcCTA CaCAGTTTaA GcGTccTGCC

              301                                                         360
PVY-D   AATTAAAGGT AGGGATATCA TCCTCATCAA AATGCCGAAA GATTTCCCTG TCTTTCCACA
120F    AATTAAGGGT AGGGACATCA TCCTCATCAA AATGCCAAAG GACTTCCCTG TTTTCCCACA
Consensus AATTAAaGGT AGGGAcATCA TCCTCATCAA AATGCCaAAa GAcTTCCCTG TcTTcCCACA

              361                                                         420
PVY-D   GAAATTGCAT TTCCGAGCTC CAACACAGAA TGAAAGAGTT TGTTTAGTTG GAACCAACTT
120F    GAAGCTACGT TTCCGAGCTC CAACACAGAA TGAGAGAATT TGTCTAGTCG GAACAAACTT
Consensus GAAacTaCaT TTCCGAGCTC CAACACAGAA TGAaAGAaTT TGTcTAGTcG GAACaAACTT

              421                                                         480
PVY-D   TCAGGAGAAG TATGCATCGT CGATCATCAC AGAGACAAGC ACCACTTACA ATATACCGGG
120F    CCAGGAGAAG TATGCATCTT CAATTGTCAC AGAAACTAGC ACCACTTACA ATGTGCCAGG
Consensus cCAGGAGAAG TATGCATCgT CaATcaTCAC AGAaACaAGC ACCACTTACA ATaTaCCaGG

              481                                                         540
PVY-D   CAGCACATTC TGGAAGCATT GGATTGAAAC AGATAATGGA CATTGTGGAC TACCAGTGGT
120F    TAGTACTTTT TGGAAGCACT GGATTGAGAC AGACGATGGG CATTGTGGAT TGCCAGTAGT
Consensus cAGcACaTTc TGGAAGCAcT GGATTGAaAC AGAcaATGGa CATTGTGGAc TaCCAGTaGT

              541                                                         600
PVY-D   GAGTACCACC GATGGATGTC TAGTCGGAAT CCACAGTTTG GCAAACAACA GACACACCAC
120F    GAGTACCGCT GATGGATGTC TACTTGGAAT ACATAGTTTG GCAAACAATG CGCAGTCCAC
Consensus GAGTACCaCc GATGGATGTC TAcTcGGAAT aCAcAGTTTG GCAAACAAca caCAcaCCAC

              601                                                         660
PVY-D   GAACTACTAC TCAGCCTTCG ATGAAGATTT TGAAAGCAAG TATCTCCGAA CCAATGAGCA
120F    GAACTACTAC TCAGCCTTTG ATGAAGATTT CGAGAGCAAA TATCTTCGAA CTAATGAGCA
Consensus GAACTACTAC TCAGCCTTcG ATGAAGATTT cGAaAGCAAa TATCTcCGAA CcAATGAGCA

              661                                                         720
PVY-D   CAATGAATGG GTCAAGTCTT GGATTTATAA TCCAGACACA GTGTTGTGGG GCCCGTTGAA
120F    TAATGAATGG ATTAAATCCT GGGTCTACAA TCCGGATACA GTGCTGTGGG GCCCACTAAA
Consensus cAATGAATGG aTcAAaTCcT GGaTcTAcAA TCCaGAcACA GTGcTGTGGG GCCCacTaAA

              721                                                         780
PVY-D   ACTTAAAGAC AGCACTCCCA AAGGATTATT CAAGACAACA AA
120F    GCTTAAAGAA AGCACTCCCA AAGGGTTGTT CAAAACAACT AAGCTTGTGC AGGATTTGGC
Consensus aCTTAAAGAa AGCACTCCCA AAGGaTTaTT CAAaACAACa AA........ ..........
```

Figure A1.4: Sequence comparison of PVY-D derived transgene and 55N PVY strain.

```
                1                                                          60
PVY-D     GTCGAACGTG AAGGACATAC CAGCACAGGA GGTGGAGCAC GAAGCTAAAT CGCTCATGAG
55N                                                                   ATGAG
Consensus ..........  .........  .........  .........  .........  .....ATGAG

               61                                                         120
PVY-D     AGGCTTGAGA GACTTTAACC CAATTGCCCA AACAGTTTGT AGGCTGAAAG TATCTGTTGA
55N       AGGTTTAAGA GATTTTAATC CAATTGCCCA AACAGTTTGT AGGCTGAAAG TATCTGTTGA
Consensus AGGcTTaAGA GAcTTTAAcC CAATTGCCCA AACAGTTTGT AGGCTGAAAG TATCTGTTGA

              121                                                         180
PVY-D     ATATGGGACA TCAGAGATGT ACGGTTTTGG ATTTGGAGCA TACATAATAG CGAACCACCA
55N       GTTTGGAACG TCAGAGTTAT ACGGTATTGG GTTTGGGGCA TACATTATAG CAAACCACCA
Consensus aTaTGGaACa TCAGAGaTaT ACGGTaTTGG aTTTGGaGCA TACATaATAG CaAACCACCA

              181                                                         240
PVY-D     TTTGTTCAGG AGTTATAATG GTTCCATGGA GGTGCGATCT ATGCACGGTA CATTCAGGGT
55N       CTTGTTCAAG AGCTACAATG GTTCAATGGA AGTGCGATCT ATGCATGGTA CATTCAGGGT
Consensus cTTGTTCAaG AGcTAcAATG GTTCaATGGA aGTGCGATCT ATGCAcGGTA CATTCAGGGT

              241                                                         300
PVY-D     GAAGAATCTA CGCAGTTTGA GCGTTCTGCC AATTAAAGGT AGGGATATCA TCCTCATCAA
55N       GAAGAACCTA CACAGTTTAA GTGTATTACC AATTAAGGGT AGGGACATCA TCCTCATCAA
Consensus GAAGAAcCTA CaCAGTTTaA GcGTacTaCC AATTAAaGGT AGGGAcATCA TCCTCATCAA

              301                                                         360
PVY-D     AATGCCGAAA GATTTCCCTG TCTTTCCACA GAAATTGCAT TTCCGAGCTC CAACACAGAA
55N       AATGCCAAAG GACTTCCCTG TTTTCCCACA GAAGCTACGT TTCCGAGCTC CAACACAGAA
Consensus AATGCCaAAa GAcTTCCCTG TcTTcCCACA GAAacTaCaT TTCCGAGCTC CAACACAGAA

              361                                                         420
PVY-D     TGAAAGAGTT TGTTTAGTTG GAACCAACTT TCAGGAGAAG TATGCATCGT CGATCATCAC
55N       TGAGAGAATT TGTCTAGTCG GAACAAACTT TCAGGAGAAG TATGCATCTT CAATTATCAC
Consensus TGAaAGAaTT TGTcTAGTcG GAACaAACTT TCAGGAGAAG TATGCATCgT CaATcATCAC

              421                                                         480
PVY-D     AGAGACAAGC ACCACTTACA ATATACCGGG CAGCACATTC TGGAAGCATT GGATTGAAAC
55N       AGAAGCTAGC ACCACTTACA ATGTGCCAGG TAGTACTTTT TGGAAGCACT GGATTGAGAC
Consensus AGAaaCaAGC ACCACTTACA ATaTaCCaGG cAGcACaTTc TGGAAGCAcT GGATTGAaAC

              481                                                         540
PVY-D     AGATAATGGA CATTGTGGAC TACCAGTGGT GAGTACCACC GATGGATGTC TAGTCGGAAT
55N       AGACGATGGG CATTGTGGAT TGCCAGTAGT GAGTACCGCT GATGGATGTC TACTTGGAAT
Consensus AGAcaATGGa CATTGTGGAc TaCCAGTaGT GAGTACCaCc GATGGATGTC TAcTcGGAAT

              541                                                         600
PVY-D     CCACAGTTTG GCAAACAACA GACACACCAC GAACTACTAC TCAGCCTTCG ATGAAGATTT
55N       ACATAGTTTG GCAAACAATG CGCAGTCCAC GAACTACTAC TCAGCCTTTG ATGAAGATTT
Consensus aCAcAGTTTG GCAAACAAca caCAcaCCAC GAACTACTAC TCAGCCTTcG ATGAAGATTT

              601                                                         660
PVY-D     TGAAAGCAAG TATCTCCGAA CCAATGAGCA CAATGAATGG GTCAAGTCTT GGATTTATAA
55N       CGAGAGCAAA TATCTCCGAA CTAATGAGCA TAATGAATGG ATTAAATCCT GGGTCTACAA
Consensus cGAaAGCAAa TATCTCCGAA CcAATGAGCA cAATGAATGG aTcAAaTCcT GGaTcTAcAA

              661                                                         720
PVY-D     TCCAGACACA GTGTTGTGGG GCCCGTTGAA ACTTAAAGAC AGCACTCCCA AAGGATTATT
55N       TCCGGATACA GTGCTGTGGG GCCCGCTAAA GCTTAAAGAA AGCACTCCCA AAGGGTTGTT
Consensus TCCaGAcACA GTGcTGTGGG GCCCGcTaAA aCTTAAAGAa AGCACTCCCA AAGGaTTaTT

              721                                                         780
PVY-D     CAAGACAACA AA
55N       TAAAACAACT AAGCTTGTGC AGGATTTGGC AGATCATGAT GTAGTAGTGG AGCAGGCCAA
Consensus cAAaACAACa AA........ .......... .......... .......... ..........
```

Appendix 2

Table A2.1: Position and methylation state of symmetric cytosines in the endogenous *CHS* gene in plants lines carrying the CHS3 hpRNA transgene.

The first column of the table describes the sequence context while the second column states the position of each analysed cytosine.

The third, fourth and fifth column describe the methylation state of cytosines in three different plant lines carrying the CHS3 hpRNA transgene. The numbers in these columns reflect the percentage of methylated cytosine residues at each given position. The data for each cytosine was obtained by analysing, on average, 20 sequences. Cells containing data for cytosines that were targeted by the CHS3 hpRNA transgenes are highlighted in grey.

The sixth column describes the methylation state of cytosines in a wild type *A. thaliana* plant.

		Plant line			
	C position	CHS 3/17	CHS 3/20	CHS 3/21	WT
CnG	-1023	0	0	0	0
CG	-1020	4	0	0	0
CG	-1006	0	0	0	4
CG	-1003	0	0	0	0
CG	-996	0	0	0	4
CnG	-994	0	0	0	0
CG	-992	0	0	0	0
CnG	-972	0	0	0	0
CG	-971	0	0	0	4
CG	-953	0	0	0	4
CnG	-940	0	0	0	4
CG	-939	0	0	0	4
CnG	-931	0	0	0	4
CG	-930	0	0	0	4
CG	-915	0	0	0	4
CG	-875	0	0	0	4
CnG	-858	0	0	0	4
CnG	-844	0	0	0	0
CG	-843	0	0	0	4
CG	-775	0	0	0	4
CG	-751	0	0	0	4
CnG	-656	0	0	0	0
CG	-655	0	0	4	0
CG	-645	0	0	4	0
CnG	-634	0	0	0	0
CnG	-625	0	0	0	4
CnG	-621	0	0	4	0
CG	-586	0	0	13	0
CG	-581	0	0	0	0
CG	-552	0	0	0	0
CG	-443	5	0	0	0
CnG	-433	5	0	0	0
CnG	-416	5	0	0	0

CG	-370	5	0	0	0
CG	-341	0	0	0	0
CG	-336	0	0	0	0
CnG	-275	27	39	65	0
CnG	-265	73	61	52	0
CG	-152	100	83	87	0
CnG	-124	45	87	87	0
CG	-98	91	100	87	0
CnG	-85	100	83	87	0
CnG	-81	100	74	83	0
CG	-80	100	83	83	0
CnG	-43	45	87	78	5
CG	-42	100	91	78	0
CnG	-37	91	74	74	0
CnG	-11	100	87	61	0
CG	-10	100	70	74	0

Table A2.2: Position and methylation state of non-symmetric cytosines in the endogenous *CHS* gene in plants lines carrying the CHS3 hpRNA transgene.

The first column of the table states the position of each analysed cytosine.

The second, third and fourth column describe the methylation state of cytosines in three different plant lines carrying the CHS3 hpRNA transgene. The numbers in these columns reflect the percentage of methylated cytosine residues at each given position. The data for each cytosine was obtained by analysing, on average, 20 sequences. Cells containing data for cytosines that were targeted by the CHS3 hpRNA transgenes are highlighted in grey.

The fifth column describes the methylation state of cytosines in a wild type *A. thaliana* plant.

	Plant line			
C position	CHS 3/17	CHS 3/20	CHS 3/21	WT
-1011	0	0	0	0
-1009	0	0	0	0
-1000	0	0	0	0
-995	0	0	0	0
-990	0	0	0	0
-986	0	0	0	0
-985	0	0	0	0
-981	0	0	0	0
-968	0	0	0	0
-934	0	0	0	4
-904	0	0	0	0
-903	0	0	0	0
-900	0	0	0	0
-899	0	0	0	0
-895	0	0	0	4
-883	0	0	0	0
-881	0	0	0	0
-879	0	0	0	0
-860	0	0	0	0
-854	0	0	0	0
-845	0	0	0	0
-840	0	0	0	0
-838	0	0	0	0
-829	0	0	0	0
-824	0	0	0	0
-823	0	0	0	0
-822	0	0	0	0
-814	0	0	0	0
-813	0	0	0	0
-797	0	0	0	0
-796	0	0	0	0
-787	0	0	0	0
-767	0	0	0	0

-760	0	0	0	4
-740	0	0	0	0
-738	0	0	0	0
-734	0	0	0	0
-733	0	0	0	0
-714	0	0	0	0
-681	0	0	0	0
-670	0	0	0	0
-667	0	0	0	0
-662	0	0	0	0
-659	0	0	0	0
-643	0	0	0	0
-615	0	0	0	4
-613	0	9	21	25
-577	0	0	0	0
-576	0	0	5	0
-567	0	0	5	0
-565	0	0	0	0
-562	0	0	0	0
-538	0	0	0	0
-521	0	0	0	0
-520	0	0	0	0
-507	0	0	0	0
-466	0	0	0	0
-445	0	0	0	0
-420	5	0	0	0
-419	5	0	0	0
-417	5	0	0	0
-400	0	0	0	0
-395	5	0	0	0
-392	10	6	0	0
-387	5	6	0	0
-366	5	0	0	0
-385	5	0	0	0
-354	5	0	0	0
-347	0	0	0	0
-345	10	6	0	0
-331	0	0	9	0
-324	0	0	9	0
-308	0	4	17	0
-304	0	9	22	0
-302	0	9	17	0
-290	9	4	22	0
-268	0	0	0	0
-266	0	17	35	0
-242	55	48	26	0
-208	73	91	87	0
-195	91	78	91	0
-191	100	91	83	0
-177	100	83	65	0
-176	100	87	74	0
-175	100	91	74	0

-173	100	83	74	0
-172	27	78	70	0
-168	100	83	70	0
-164	91	87	74	0
-136	100	87	83	0
-127	100	91	83	0
-126	45	87	78	0
-112	100	74	70	0
-108	100	78	74	0
-105	100	65	74	0
-100	27	74	65	0
-91	100	83	61	0
-88	100	70	65	0
-82	100	87	70	0
-77	100	61	57	0
-76	100	83	70	0
-73	100	78	52	0
-69	100	61	65	0
-68	100	70	61	0
-65	100	70	52	0
-64	100	78	43	0
-62	100	48	57	0
-60	100	70	52	0
-58	100	78	57	0
-56	100	78	70	0
-53	73	87	70	5
-50	100	87	61	5
-44	45	87	83	0
-21	45	74	52	0
-19	45	57	70	0
-17	45	70	52	0
-15	45	70	57	0
-13	45	65	52	0

Table A2.3: Position and methylation state of symmetric cytosines in the endogenous *CHS* gene in plants lines carrying the CHS2 hpRNA transgene.

The first column of the table describes the sequence context while the second column states the position of each analysed cytosine.

The third, fourth and fifth column describe the methylation state of cytosines in three different plant lines carrying the CHS2 hpRNA transgene. The numbers in these columns reflect the percentage of methylated cytosine residues at each given position. The data for each cytosine was obtained by analysing, on average, 20 sequences. Cells containing data for cytosines that were targeted by the CHS2 hpRNA transgenes are highlighted in grey.

The sixth column describes the methylation state of cytosines in a wild type *A. thaliana* plant.

		Plant line			
	C position	CHS 2/2	CHS 2/8	CHS 2/11	WT
CnG	-1023	0	0	0	0
CG	-1020	0	0	4	0
CG	-1006	0	0	0	4
CG	-1003	0	0	0	0
CG	-996	0	0	0	4
CnG	-994	0	0	0	0
CG	-992	4	0	0	0
CnG	-972	0	0	0	0
CG	-971	0	0	0	4
CG	-953	0	0	0	4
CnG	-940	0	0	0	4
CG	-939	0	0	0	4
CnG	-931	0	0	0	4
CG	-930	0	0	0	4
CG	-915	0	0	0	4
CG	-875	0	0	0	4
CnG	-858	0	0	0	4
CnG	-844	0	0	0	0
CG	-843	0	0	0	4
CG	-775	0	0	0	4
CG	-751	0	0	0	4
CnG	-656	4	4	0	0
CG	-655	0	4	0	0
CG	-645	4	0	0	0
CnG	-634	0	0	5	0
CnG	-625	4	13	9	4
CnG	-621	0	0	5	0
CG	-586	8	8	5	0
CG	-581	0	17	0	0
CG	-552	0	0	0	0
CG	-443	25	67	14	0
CnG	-433	42	88	23	0
CnG	-416	21	83	23	0

CG	-370	54	92	27	0
CG	-341	58	96	32	0
CG	-336	54	92	36	0
CnG	-275	100	86	100	0
CnG	-265	96	91	100	0
CG	-152	83	91	100	0
CnG	-124	71	68	100	0
CG	-98	42	82	100	0
CnG	-85	38	45	100	0
CnG	-81	42	59	100	0
CG	-80	46	73	9	0
CnG	-43	13	18	91	5
CG	-42	25	41	91	0
CnG	-37	21	55	100	0
CnG	-11	13	14	0	0
CG	-10	8	18	0	0

Table A2.4: Position and methylation state of non-symmetric cytosines in the endogenous *CHS* gene in plants lines carrying the CHS2 hpRNA transgene.

The first column of the table states the position of each analysed cytosine.

The second, third and fourth column describe the methylation state of cytosines in three different plant lines carrying the CHS2 hpRNA transgene. The numbers in these columns reflect the percentage of methylated cytosine residues at each given position. The data for each cytosine was obtained by analysing, on average, 20 sequences. Cells containing data for cytosines that were targeted by the CHS2 hpRNA transgenes are highlighted in grey.

The fifth column describes the methylation state of cytosines in a wild type *A. thaliana* plant.

	Plant line			
C position	**CHS 2/2**	**CHS 2/8**	**CHS 2/11**	**WT**
-1011	0	0	0	0
-1009	0	0	0	0
-1000	0	0	0	0
-995	0	0	0	0
-990	0	0	0	0
-986	0	0	0	0
-985	0	0	0	0
-981	0	0	0	0
-968	0	0	0	0
-934	0	0	0	4
-904	0	0	0	0
-903	0	0	0	0
-900	0	0	0	0
-899	0	0	0	0
-895	0	0	0	4
-883	0	0	0	0
-881	0	0	0	0
-879	0	0	0	0
-860	0	0	0	0
-854	0	0	0	0
-845	0	0	0	0
-840	0	0	0	0
-838	0	0	0	0
-829	0	0	0	0
-824	0	0	0	0
-823	0	0	0	0
-822	0	0	0	0
-814	0	0	0	0
-813	0	0	0	0
-797	0	0	0	0
-796	0	0	0	0
-787	0	0	0	0

-767	0	0	0	0
-760	0	0	0	4
-740	0	0	0	0
-738	0	0	0	0
-734	0	0	0	0
-733	0	0	0	0
-714	0	0	0	0
-681	0	0	0	0
-670	0	0	5	0
-667	0	0	0	0
-662	8	21	5	0
-659	0	0	0	0
-643	4	0	0	0
-615	0	0	0	4
-613	8	0	0	25
-577	0	0	0	0
-576	0	0	0	0
-567	0	0	0	0
-565	0	8	0	0
-562	0	0	0	0
-538	4	0	0	0
-521	0	8	5	0
-520	0	8	5	0
-507	13	8	5	0
-466	0	17	0	0
-445	17	25	14	0
-420	13	33	18	0
-419	13	50	18	0
-417	13	46	14	0
-400	38	50	14	0
-395	33	50	18	0
-392	42	63	18	0
-387	25	50	9	0
-366	29	50	9	0
-385	25	46	14	0
-354	21	13	14	0
-347	17	13	18	0
-345	25	33	18	0
-331	83	68	91	0
-324	75	64	100	0
-308	75	68	100	0
-304	67	64	9	0
-302	75	45	100	0
-290	67	50	100	0
-268	0	0	0	0
-266	25	32	100	0
-242	71	45	100	0
-208	71	50	100	0
-195	71	68	100	0
-191	71	64	100	0
-177	63	68	100	0
-176	67	64	100	0

-175	63	77	100	0
-173	58	73	100	0
-172	58	73	100	0
-168	75	73	100	0
-164	54	55	100	0
-136	71	55	100	0
-127	42	36	100	0
-126	54	32	100	0
-112	58	9	100	0
-108	33	0	100	0
-105	42	9	100	0
-100	25	9	100	0
-91	25	18	100	0
-88	17	18	91	0
-82	33	36	91	0
-77	17	14	100	0
-76	0	18	100	0
-73	33	36	73	0
-69	4	9	82	0
-68	38	18	55	0
-65	21	14	100	0
-64	29	9	0	0
-62	29	14	91	0
-60	25	14	0	0
-58	25	18	0	0
-56	25	27	0	0
-53	25	14	91	5
-50	17	14	91	5
-44	13	18	91	0
-21	8	0	0	0
-19	8	0	0	0
-17	4	5	0	0
-15	17	5	0	0
-13	0	0	0	0

Table A2.5: Position and methylation state of symmetric cytosines in the endogenous *CHS* gene in plants lines carrying the CHS1 hpRNA transgene.

The first column of the table describes the sequence context while the second column states the position of each analysed cytosine.

The third, fourth and fifth column describe the methylation state of cytosines in three different plant lines carrying the CHS1 hpRNA transgene. The numbers in these columns reflect the percentage of methylated cytosine residues at each given position. The data for each cytosine was obtained by analysing, on average, 20 sequences. Cells containing data for cytosines that were targeted by the CHS1 hpRNA transgenes are highlighted in grey.

The sixth column describes the methylation state of cytosines in a wild type *A. thaliana* plant.

		Plant line			
	C position	**CHS 1/3**	**CHS 1/10**	**CHS 1/15**	**WT**
CnG	-1023	4	14	0	0
CG	-1020	25	18	13	0
CG	-1006	54	32	39	4
CG	-1003	79	36	35	0
CG	-996	71	64	52	4
CnG	-994	29	0	6	0
CG	-992	83	64	55	0
CnG	-972	42	14	3	0
CG	-971	88	68	61	4
CG	-953	100	82	81	4
CnG	-940	50	23	26	4
CG	-939	100	86	81	4
CnG	-931	71	45	52	4
CG	-930	100	82	90	4
CG	-915	100	82	90	4
CG	-875	96	82	100	4
CnG	-858	83	86	77	4
CnG	-844	63	55	42	0
CG	-843	100	77	94	4
CG	-775	88	86	94	4
CG	-751	79	86	90	4
CnG	-656	30	46	35	0
CG	-655	91	88	91	0
CG	-645	91	96	91	0
CnG	-634	39	21	30	0
CnG	-625	61	54	65	4
CnG	-621	65	71	70	0
CG	-586	91	92	100	0
CG	-581	87	96	91	0
CG	-552	76	58	61	0
CG	-443	67	83	78	0
CnG	-433	57	67	78	0
CnG	-416	71	92	87	0

CG	-370	86	92	78	0
CG	-341	100	83	91	0
CG	-336	81	83	96	0
CnG	-275	90	85	91	0
CnG	-265	100	85	82	0
CG	-152	81	100	91	0
CnG	-124	81	46	64	0
CG	-98	76	54	50	0
CnG	-85	43	23	55	0
CnG	-81	57	23	41	0
CG	-80	81	31	64	0
CnG	-43	33	0	23	5
CG	-42	38	38	64	0
CnG	-37	19	54	36	0
CnG	-11	48	0	9	0
CG	-10	48	0	27	0

Table A2.6: Position and methylation state of non-symmetric cytosines in the endogenous *CHS* gene in plants lines carrying the CHS1 hpRNA transgene.

The first column of the table states the position of each analysed cytosine.

The second, third and fourth column describe the methylation state of cytosines in three different plant lines carrying the CHS1 hpRNA transgene. The numbers in these columns reflect the percentage of methylated cytosine residues at each given position. The data for each cytosine was obtained by analysing, on average, 20 sequences. Cells containing data for cytosines that were targeted by the CHS1 hpRNA transgenes are highlighted in grey.

The fifth column describes the methylation state of cytosines in a wild type *A. thaliana* plant.

	Plant line			
C position	**CHS 1/3**	**CHS 1/10**	**CHS 1/15**	**WT**
-1011	25	0	0	0
-1009	29	0	0	0
-1000	38	0	0	0
-995	33	0	3	0
-990	29	5	3	0
-986	33	5	3	0
-985	46	5	10	0
-981	42	5	3	0
-968	46	18	13	0
-934	33	18	19	4
-904	25	36	23	0
-903	33	45	35	0
-900	33	41	32	0
-899	42	45	32	0
-895	54	41	35	4
-883	33	36	26	0
-881	38	32	19	0
-879	33	23	13	0
-860	50	36	19	0
-854	50	36	26	0
-845	50	18	35	0
-840	58	23	32	0
-838	29	14	29	0
-829	50	27	45	0
-824	29	18	29	0
-823	38	23	42	0
-822	29	18	35	0
-814	21	23	32	0
-813	42	23	42	0
-797	21	27	29	0
-796	46	32	39	0
-787	42	36	26	0

-767	38	36	26	0
-760	63	50	45	4
-740	13	21	22	0
-738	13	17	26	0
-734	13	13	26	0
-733	22	29	30	0
-714	9	13	4	0
-681	13	17	17	0
-670	0	8	9	0
-667	4	13	4	0
-662	0	4	4	0
-659	0	8	9	0
-643	52	25	22	0
-615	9	8	9	4
-613	43	21	30	25
-577	5	8	4	0
-576	19	17	4	0
-567	0	8	4	0
-565	48	42	17	0
-562	10	8	0	0
-538	0	8	0	0
-521	0	0	0	0
-520	29	8	17	0
-507	5	8	13	0
-466	10	8	0	0
-445	5	8	4	0
-420	5	0	0	0
-419	5	8	0	0
-417	5	0	0	0
-400	14	0	9	0
-395	19	17	9	0
-392	57	50	30	0
-387	0	0	0	0
-366	19	0	0	0
-385	29	8	17	0
-354	5	17	9	0
-347	10	8	13	0
-345	24	25	9	0
-331	62	46	55	0
-324	76	54	64	0
-308	67	31	45	0
-304	52	62	55	0
-302	62	62	59	0
-290	33	46	59	0
-268	0	0	0	0
-266	33	15	18	0
-242	43	69	23	0
-208	57	54	45	0
-195	52	46	23	0
-191	71	38	32	0
-177	43	31	32	0
-176	57	31	36	0

-175	52	38	41	0
-173	62	31	32	0
-172	57	31	32	0
-168	67	23	45	0
-164	48	8	23	0
-136	52	62	50	0
-127	33	38	32	0
-126	24	62	27	0
-112	38	38	14	0
-108	43	15	23	0
-105	33	15	18	0
-100	33	0	14	0
-91	19	15	18	0
-88	14	0	14	0
-82	19	23	27	0
-77	10	0	0	0
-76	10	0	0	0
-73	38	8	9	0
-69	10	0	0	0
-68	29	0	14	0
-65	10	15	5	0
-64	33	8	9	0
-62	14	0	5	0
-60	33	8	0	0
-58	33	0	9	0
-56	29	0	5	0
-53	24	0	0	5
-50	19	0	0	5
-44	14	8	0	0
-21	33	0	0	0
-19	48	0	9	0
-17	19	0	9	0
-15	24	0	9	0
-13	14	8	5	0

Table A2.7: Position and methylation status of symmetric cytosines in the endogenous *CHS* gene and the CHS400 transgene in plants lines carrying CHS100 or CHS400 hpRNA transgene.

The first column of the table describes the sequence context while the second column states the position of each analysed cytosine.

The third column describes the methylation status of cytosines in the endogenous *CHS* gene in a plant line carrying the CHS100 hpRNA transgene. The fourth column describes the methylation status of cytosines in the endogenous *CHS* gene in a plant line carrying the CHS400 hpRNA transgene. The fifth column describes the methylation status of cytosines in the CHS400 hpRNA transgene. Data for some cytosine residues within the transgene are not available as only one bisulfite PCR product was used to analyse the methylation status of the transgene. The sixth column describes the methylation status of cytosines in a wild type *A. thaliana* plant. The numbers in these columns reflect the percentage of methylated cytosine residues at each given position.

The data for each cytosine was obtained by analysing, on average, 20 sequences. Cells containing data for cytosines that were targeted by the CHS100 or CHS400 hpRNA transgene are highlighted in grey. Cells containing data for cytosines located within the *CHS* intron are highlighted in pink.

	C position	CHS 100 (endogene)	CHS 400 (endogene)	CHS 400 (transgene)	WT
CnG	-11	0	0		0
CG	-10	0	0		0
CnG	31	0	0		0
CnG	85	0	0		0
CnG	110	0	0		0
CnG	114	0	0		0
CnG	120	0	0		0
CnG	127	0	0		0
CnG	137	5	11		0
CnG	139	0	11		0
CnG	160	20	32		0
CnG	169	25	37		0
CnG	186	20	42		0
CG	190	15	32		0
CnG	212	20	37		0
CG	213	30	53		0
CnG	224	5	42	50	0
CnG	238	20	37	39	0
CG	239	10	42	78	0
CG	261	5	47	72	0
CG	271	0	16		0
CnG	351	0	11		0
CG	355	5	6	78	0
CG	363	0	6	89	0

CG	371	5	28	83	0
CG	377	0	17	56	0
CnG	389	0	22	78	0
CG	393	0	22	83	0
CnG	446	0	17	50	0
CnG	454	0	28	67	0
CnG	458	0	17	50	0
CG	466	0	11	78	0
CG	475	0	28	83	0
CG	501	0	28	89	0
CnG	504	0	11	50	0

Table A2.8: Position and methylation status of non-symmetric cytosines in the endogenous *CHS* gene and the CHS400 transgene in plants lines carrying CHS100 or CHS400 hpRNA transgene.

The first column of the table states the position of each analysed cytosine.

The second column describes the methylation status of cytosines in the endogenous *CHS* gene in a plant line carrying the CHS100 hpRNA transgene. The third column describes the methylation status of cytosines in the endogenous *CHS* gene in a plant line carrying the CHS400 hpRNA transgene. The fourth column describes the methylation status of cytosines in the CHS400 hpRNA transgene. Data for some cytosine residues within the transgene are not available as only one bisulfite PCR product was used to analyse the methylation status of the transgene. The fifth column describes the methylation status of cytosines in a wild type *A. thaliana* plant. The numbers in these columns reflect the percentage of methylated cytosine residues at each given position.

The data for each cytosine was obtained by analysing, on average, 20 sequences. Cells containing data for cytosines that were targeted by the CHS100 or CHS400 hpRNA transgene are highlighted in grey. Cells containing data for cytosines located within the *CHS* intron are highlighted in pink.

C position	CHS 100 (endogene)	CHS 400 (endogene)	CHS 400 (transgene)	WT
1	0	0		0
2	0	0		0
8	5	0		0
10	0	0		0
11	0	0		0
15	0	0		0
26	0	0		0
28	0	0		0
34	0	0		0
37	0	0		0
40	0	0		0
44	0	0		0
46	0	0		0
54	0	0		0
55	0	0		0
56	0	0		0
70	0	0		0
91	0	0		0
94	0	0		0
97	0	0		0
118	0	0		0
136	0	0		0
143	5	0		0
145	10	11		0
151	10	16		0

158	10	16		0
163	25	16		0
167	20	16		0
168	15	21		0
176	20	26		0
177	10	32		0
183	20	21		0
198	15	32		0
199	10	37		0
203	15	26		0
206	20	42		0
209	15	32		0
215	5	26		0
218	10	26		0
220	15	26		0
221	20	32		0
231	15	26	6	0
233	20	32	6	0
242	10	32	6	0
243	5	21	6	0
245	5	26	33	0
257	5	21	22	0
263	0	5	11	0
274	0	0		0
282	0	0		0
285	0	0		0
288	0	0		0
292	0	0		0
297	0	0		0
298	0	0		0
304	0	0		0
311	0	0		0
314	0	0		0
318	0	0		0
320	0	6		0
330	0	6		0
334	0	11		0
345	0	6		0
349	0	6		0
358	0	6	44	0
366	0	6	33	0
380	0	6	28	0
382	0	6	22	0
386	0	6	28	0
403	0	0	22	0
404	0	0	28	0
406	0	0	50	0
415	0	0	17	0
416	0	0	28	0
417	0	0	33	0
419	0	0	17	0
421	0	0	22	0

429	0	0	28	0
433	0	0	22	0
438	0	0	17	0
440	0	0	22	0
441	0	0	28	0
444	0	0	33	0
451	0	0	22	0
453	0	0	28	0
463	0	0	22	0
481	0	0	11	0
482	0	6	17	0
483	0	11	17	0
488	0	0	17	0
493	0	0	28	0

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